| FORM PTO-1390 (REV 11-2000) | U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | ATTORNEY'S DOCKET NUMBER 2370-67 |
|---|---|---|
| **TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371** | | U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/936786 Unassigned |
| INTERNATIONAL APPLICATION NO. **PCT/US00/06940** | INTERNATIONAL FILING DATE **17 March 2000** | PRIORITY DATE CLAIMED **18 March 1999** |

TITLE OF INVENTION

**CELLS, CELL POPULATIONS, AND METHODS OF MAKING AND USING SAME**

APPLICANT(S) FOR DO/EO/US

**MAJOR et al**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☐ A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11 To 20 below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information.

| U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5) | INTERNATIONAL APPLICATION NO | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| Unassigned 09/936786 | PCT/US00/06940 | 2370-67 |

21. ☒ The following fees are submitted:

| CALCULATIONS | PTO USE ONLY |
|---|---|

**BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):**

-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .....$1000.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO .....$860.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO .....$710.00

-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) .....$690.00

-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) .....$100.00

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|---|---|---|---|---|---|---|---|---|
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | | | | | | $ | 690.00 | |
| Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)). | | | | | | $ | 130.00 | |
| CLAIMS | NUMBER FILED | | NUMBER EXTRA | RATE | | | | |
| Total Claims | 34 | -20 = | 14 | X | $18.00 | $ | 252.00 | |
| Independent Claims | 4 | -3 = | 1 | X | $80.00 | | 80.00 | |
| MULTIPLE DEPENDENT CLAIMS(S) (if applicable) | | | | | $270.00 | $ | 0.00 | |
| **TOTAL OF ABOVE CALCULATIONS =** | | | | | | $ | **1152.00** | |
| ☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. | | | | | | | 0.00 | |
| **SUBTOTAL =** | | | | | | $ | **1152.00** | |
| Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). + | | | | | | | 0.00 | |
| **TOTAL NATIONAL FEE =** | | | | | | $ | **1152.00** | |
| Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property + | | | | | | $ | 0.00 | |
| Fee for Petition to Revive Unintentionally Abandoned Application ($1240.00 – Small Entity = $620.00) | | | | | | $ | 0.00 | |
| **TOTAL FEES ENCLOSED =** | | | | | | $ | **1152.00** | |
| | | | | | | Amount to be: refunded | $ | |
| | | | | | | Charged | $ | |

a. ☒ A check in the amount of $1152.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.

d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

**NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

**SEND ALL CORRESPONDENCE TO:**

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**29,009** September 18, 2001
REGISTRATION NUMBER Date



559019

# CELLS, CELL POPULATIONS, AND METHODS OF MAKING AND USING SAME

The present application claims benefit of U. S. Provisional Patent Application No. 60/124,889, filed March 18, 2000, the entire contents of which is hereby incorporated by reference.

An invention described herein was made by an agency of the United States Government or under contract with an agency of the United States Government. The name of the U. S. Government agency and the Government contract number are: NIH - CRADA No. 94-003-NS.

## BACKGROUND OF THE INVENTION

The present invention generally relates to stem cells, including progenitor and precursor cells, which are preferably immortalized, and have been derived from the human fetal central nervous system (CNS); methods for treating a host by implanting the disclosed cells, and genetically altered forms of the disclosed cells in the host. More particularly, the present invention provides CNS derived cell lines, such as fetal CNS derived cell lines, such as human fetal CNS derived cell lines, and methods of treating a host by implantation of these immortalized CNS derived cells into the host. Also disclosed are methods of isolating immortalized CNS derived cells useful in therapeutic applications.

Cell transplant therapy is particularly appealing for treatment of neurological diseases. Solid tissue transplantation is especially inappropriate for neurological diseases for several reasons. Open surgical exposure of the brain, as required for solid tissue transplantation, can cause irreparable damage to nervous system pathways resulting in clinical neurological deficits. Also, neurological function often depends on complex intercellular connections that can not be surgically established. Further, cells of the central nervous system are exquisitely sensitive to anoxia and nutrient deprivation. Rapid vascularization of solid tissue transplants is critical as cells in the interior of solid tissue transplants often lack sufficient perfusion to maintain viability. Stenevi et al., Brain Res., 114:1-20 (1976).

One common neurological syndrome, Parkinsonism has been the object of attempts at cell transplant therapy. Bjorklund et al.,Brain Res., 177:555-560 (1979); Lindvall et al., Science, 247:574-577 (1990); Freed, Restor. Neurol. Neurosci., 3:109-134 (1991). Parkinsonism is caused by a loss of dopamine-producing neurons in the substantia nigra of the basal ganglia. Burns et al.,

N. Engl. J. Med., 312:1418-1421 (1985); Wolff et al., Neurobiology, 86:9011-9014 (1989). Parkinson's disease, a disease of unknown etiology that is characterized by the clinical manifestations of Parkinsonism, is caused by idiopathic destruction of these dopamine-producing neurons. Parkinsonism may be caused by a variety of drugs, e.g., antipsychotic agents, or chemical agents, e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Burns et al., Proc. Natl. Acad. Sci. USA, 80:4546-4550 (1983) and Bankiewicz et al., Life Sci., 39:7-16 (1986).

Attempts have been made to reverse the clinical manifestations of experimentally-induced Parkinsonism by transplanting dopaminergic cells into the striatum of affected animals. Genetically modified fibroblasts (transfected with DNA encoding tyrosine hydroxylase) have been successfully transplanted into animals having lesions of dopaminergic pathways. Motor function and behavior of the animals improved following implantation of the dopamine producing fibroblasts. Wolff et al., Proc. Natl. Acad. Sci. USA, 86:9011-9014 (1989); Fisher et al., Neuron, 6:371-380 (1991). Graft survival may be enhanced, and hence clinical improvement prolonged, by transplantation of fetal tissue, as compared to cells obtained following birth. Gage and Fisher, Neuron, 6:1-12 (1991). Fresh fetal dopaminergic neurons have been transplanted into the caudate nucleus of monkeys following chemical injury to the nigrostriatal dopamine system. Following transplantation, the injury-induced behavioral deficits improved. Bankiewicz et al., J. Neurosurg., 72:231-244 (1990) and Taylor et al., Prog. Brain Res., 82:543-559 (1990).

Humans suffering from Parkinsonism have been treated by striatal implantation of dopaminergic human fetal neurons. Lindvall et al., Arch. Neurol., 46:615-631 (1989); Widner et al., New Engl. J. Med., 327:1556-1563 (1992). The transplanted cells were obtained from abortions. Prior to the abortions, the women were screened for antibodies to several disease causing viruses. Following surgery, the treated patients exhibited improvement of neurological function. The patients required maintenance immunosuppressive therapy, however.

Recent investigations indicate that trophic factors released from support cells of the central nervous system (e.g., astrocytes and oligodendrocytes) are critical to survival of neurons in cell culture. O'Malley et al., Exp. Neurol., 112:40-48 (1991). Implanted fibroblasts that were genetically altered to express nerve growth factor have been shown to enhance survival of cholinergic neurons of the basal forebrain following injury to the fimbria-fornix which causes demise of acetylcholine neurons in the basal forebrain as seen in Alzheimer's disease. Rosenberg et al., Science, 242:1575-1577 (1988).

While previous attempts at cell transplant therapy for neurological disorders have provided encouraging results, several significant problems remain. The supply of fetal tissue for cellular transplants is quite limited. To ensure maximum viability, the fetal cells must be freshly harvested prior to transplantation. This requires coordinating the implantation procedure with elective abortions. Even then, fetal tissue has not been widely available in the United States. Also, the gestational age of the fetus from which cells are obtained influences graft survival. Gage and Fisher, supra. Obtaining fetal tissue of only certain gestational ages adds additional limitations to the availability of fetal cells for transplant. Further, ethical considerations make some potential transplant recipients reluctant to undergo the procedure when fresh fetal cells are implanted.

Because the fetal tissue is obtained from fresh abortuses, a significant risk of infectious contamination exists. Although women undergoing abortions that will supply fetal tissue are screened for a variety of infections, some infections, e.g., HIV, may not be clinically detectable and thus, not identified during the screening process. Therefore, if widely practiced, transplants of fresh fetal cells would likely cause many infectious sequelae.

Use of immortalized cell lines could overcome many of these difficulties of availability and infection. An immortalized human fetal neuro-derived cell line containing an immortalizing gene has been reported in Major et al., Proc. Natl. Acad. Sci. USA, 82:1257-1262 (1985) and U.S. Pat. No. 4,707,448.

Recently, it has been found that a cell line which was produced by transfecting a population of human fetal derived cells from the central nervous system with an origin-defective mutant of SV40 virus, which are referred to as SVG cells, have shown promise in treatment of neurological transplantation therapy. See, U.S. Patent Nos. 5,753,491; 5,869,463 and 5,690,927. The SVG cells have been described as an example of a permanently established line of fetal glial cells. See, . Major *et al.*, Proc Natl Acad Sci U S A, 82: 1257-61 (1985), which was the first report of the use of an immortalizing gene to produce potentially immortal cells of the human central nervous system. This population of SVG cells has also been characterized as glial or neuro-glial . Fitoussi *et al.*, Neuroscience, 85: 405-13 (1998); Major *et al.*, Proc Natl Acad Sci U S A, 82: 1257-61 (1985); Tornatore *et al.*, Cell Transplant, 5: 145-63 (1996) and issued patents number 4,707,448; 5,690,927; 5,753,491; 5,869,463. The SVG cells have been propagated in "standard media" that included EMEM (Eagle's Minimum Essential Medium + 10% fetal bovine serum (FBS) + 2 mM l-glutamine). The SVG cell line can be readily transfected by standard

methods and express heterologous DNA . Tornatore *et al.*, Cell Transplant, 5: 145-63 (1996) and U.S. Patent No. 5,753,491, which may include biologically active molecules useful in treatment of neurological disorders.

More recently, it has been appreciated that the CNS, like the hematopoietic system, contains cells that are capable of developing into more phenotypically defined cells. That is, it is now more readily appreciated that the CNS contains progenitor or stem cells which, if purified, maintained and stored, may contain the potential to develop a transplantable product which could be developed, either pre- or post-transplantation, into multiple cell types, for multiple uses. These cells would be, therefore, multipotent and may reduce the requirement for production and maintenance of multiple cell types. Moreover, transplanting such a multipotent cell may allow replacement and re-population of the cell type needed by the patient, without need for external effectors as the cells may be able to develop to replace lost or damaged tissue types.

What is urgently needed therefore in the art are methods of therapeutically implanting immortalized human fetal CNS-derived stem or multipotent cells, and cell lines suitable for this use. Ideally, the methods would not result in tumor formation or elicit intense inflammation following transplantation. Desirably, the methods could employ cells derived from cell lines so that the risk of infectious contamination and limited cellular availability would be minimized. Quite surprisingly, the present invention fulfills these and other related needs.

## SUMMARY OF THE INVENTION

The present invention provides methods for treating a host comprising implanting multipotent cells of an immortalized human neuro-derived cell line, preferably a fetal cell line, into the host. The cells for implantation and methods of their identification and purification are also provided. Generally the cell line, and cell types within those cell lines, will be derived from CNS cells, such as human fetal CNS cells, such as the SVG cell line. The cells may be implanted into the central nervous system of the host without further differentiation into either specifically a neural or glial cell type. Alternatively, the multipotent cells of the present invention may be further differentiated prior to use in the treatment methods of the present invention. The cells may be encapsulated by membranes which are impermeable to antibodies of the host.

In some embodiments of the invention, the multipotent cells may be transfected with a nucleic acid sequence encoding a peptide, amino acid sequence or protein. The peptides, amino acid sequences, or proteins will generally be enzymes, such as tyrosine hydroxylase, or growth

factors, such as nerve growth factor, or portions thereof, including glycosylated and non-glycosylated peptides and proteins. The peptide, amino acid sequences and proteins, hereinafter generally referred to by any one of these terms, may also be a disease-associated antigen. The cells may be implanted for purposes of treatment or prophylaxis. In some instances, the cells may be removed following implantation.

In additional embodiments, the present invention provides a multipotent immortalized human fetal CNS-derived cell line, and specific cell types derived from these cell lines, which contains a heterologous nucleic acid sequence, wherein the cell line is capable of expressing the heterologous nucleic acid sequence.

Particularly preferred cell lines and cell types are capable of expressing a nucleic acid that encodes tyrosine hydroxylase, serotonin or aromatic amino acid decarboxylase.

In a related embodiment, the present invention provides a transplantable composition that contains at least one of the multipotent cell types of the invention, or derivatives thereof, with a pharmaceutically acceptable carrier.

The present invention provides therefore an isolated fetal central nervous system cell line containing a multipotent cell that has the ability to divide, without limit, and the potential to differentiate toward a neuronal cell or a glial cell. The cell line of the present invention perferably contains a stem, progenitor or precursor cell. The fetal central nervous system derived cell line is derived from a human fetal central nervous system.

The present invention provides a cluster of cells, preferably isolated and/or purified, of the invention, preferably in the form of a neurosphere.

In one embodiment, the present invention provides an isolated and/or purified multipotent cell. The cells of the present invention may be characterized by any of the following marker combinations: TnTx-/ChTx-, TnTx+/ChTx+, TnTx+/ChTx-, TnTx+/ChTx+, A2B5-/TnTx-, A2B5+/TnTx-, A2B5-/TnTx+, A2B5-/ChTx-, A2B5+/ChTx+, A2B5-/ChTx+, A2B5+/ChTx-, TnTx-/ChTx-/nestin+, and/or TnTx-/ChTx-/nestin-, as further described and defined herein.

The present invention further providfes an isolated and/or purified cell and/or tissue derived from the cell line of the present invention. The cells and/or tissues of the present invention may further contain a heterologous nucleic acid sequence which encodes a biologically active peptide or protein which may be, for example, a disease associated peptide or protein, an enzyme, a trophic factor, and/or a cytokine. The enzymes encoded in the cells and/or tissues of the present invention may be, for example, tyrosine hydroxylase, GTPCH1, AADC or VMAT2.

The trophic factors encoded for may be, for example, GDNF, VEGF, BDNF, NGF, bFGF, TGFΞ (including the TGFΞ family of peptides), CNTF, PDGF, BMP, LIF, Neurturin, Persephin, Neublastin, NT4/5, NT3, or Midkine. The cytokine may be, for example, IL-10 or IL-6. The heterologous nucleic acid may be operably linked to a transcriptional promoter, which may be, for example, a regulatable promoter.

The prepsent invention provides populations of cells defined herein as NG1, NG2, and NG3 populations of cells.

The present invention provides a method of identifying a multipotent cell which includes measuring for the presence or absence of the binding partners for TnTx and ChTx and the A2B5 antibody in a cell sample which is believed to contain a multipotent cell. The method of the present invention may be used to identify multipotent cells derived from the fetal central nervous system derived cells. Specifically, the present invention provides a method wherein these cells are identified by mixing a sample containing the same with at least one factor which specifically binds to at least one cell-specific binding partner selected from an A2B5 antibody-binding cell binding partner, TnTx receptor, and ChTx receptor, or fragments thereof, under conditions where the factor binds to the cell, followed by detecting of the binding, as an indication of the presence of the multipotent cell. The factors used in the methods of the present invention include an A2B5 antibody or fragment, a ChTx or a fragment thereof, and a TnTx or a fragment thereof. One of ordinary skill will appreciate that binding partners and factors in this context may include an antibody, an antibody fragment, a ligand, a ligand fragment, a receptor or receptor fragment.

The method of the present invention may also include identifying the cells by their ability to specifically bind to a human nestin antibody.

Moreover, the binding partner, factors, ligands and antibodies used for detection in the present method may include or contain a detectable label which may be, for example, fluorescent, chemiluminescent, radioactive, immunologically detectable, and/or an enzymatically active component of a detection system.

The method of identifying cells according to the present invention may include analyzing the cells with a fluorescence activated cell sorter which may also be used to purify, enrich and/or separate specific cell populations.

The present invention provides a method of enriching a population of cells the multipotent fetal nervous system derived cells which includes culturing the population in the

presence of serum, preferably through crisis, followed by culturing the population in a non-serum containing media.

Moreover, the present invention provides a method of treating a mammal having a neurological syndrome or disease which includes implanting in to the mammal a therapeutically effective amount of a composition containing at least one cell or cells population of the present invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Immunoidentification of SVG P50 cells cultured in fetal calf serum-containing or serum-free media is shown wherein a comparison is provided between control and forskolin-treatment. In this example, analysis was gated on live cells. Triple label characterization of live NG1 and NG3 cells is shown. Results of fluorescent emission of labeled NG1 cells are shown in panels A-F (panels A-C being control and panels D-F being forskolin treated) and labeled NG3 cells are shown in in panels G-L (panels G-I being controls and panels J-L being forskolin treated). Panels A, D, G and J show show results of A2B5-phycoerytrin (PE) labeling versus TnTx -TC labeling and panels B, E, H and K show results of ChTx-FITC labeling versus TnTx-TC labeling, and panels C, F, I and L show results of ChTx-FITC labeling versus A2B5-PE labeling. Each panel is divided into four sections or quadrants wherein each section is designated as UL (upper left), UR (upper right), LL (lower left) and LR (lower right) with the number of positive cells for each section shown to the right of the panel. Upper left (UL) indicates cells that were negative for the X-axis label and negative for the Y-axis label. Upper right (UR) indicates cells that were positive for both the X- and Y-axis labels. Lower left (LL) indicates cells that were negative for both X- and Y-axis labels. Lower right (LR) indicates cells that were positive for the X-axis label and negative for the Y-axis label. Figure 1 shows the effect of serum culture medium (NG1 cells) compared to the Neurobalsal + N2 medium (NG3 cells) on the expression of the cell surface markers. NG3 cells had an increase in TnTx+ cells (UL in A versus G) and a decrease in A2B5+ cells (LR in A versus G) compared to the NG1 cells. Notably, there was no major change due to forskolin in the NG1 or NG3 cells. Specifically, there was no increase in the TnTx+ cells (UR in B versus E and H versus K) indicating that there was no increase in the neuronal lineage cells due to forskolin.

Figure 2: Immunoidentification of SVG P50 cells cultured in serum-free media with anti-ChTx-FITC, anti-TnTx-PE/Cy5 and anti-nestin-PE. Triple label characterization of NG3 cells was performed with labels for ChTx, TnTx and nestin. Panels A-C indicate the relative intensity of the fluorescent signal for each marker (TnTx, ChTx or nestin) individually. Therefore, there are predominantly ChTx+ cells (A), rare nestin+ cells (B) and mostly TnTx- cells (C) observed in the general population of NG3 cells. Panels D-F show results of live cells labeled with the indicated markers (i.e., panel D showing TnTx-TC (fragment C) labeling versus nestin-PE labeling; panel E showing TnTx-TC and ChTx-FITC labeling; and paenl F showing nestin-PE and ChTx-FITC labeling) wherein the percent of cells in each quadrant, as described above in relation to Figure 1, is provided to the left of each panel. Examination of panels G-K indicates that the nestin+ cells have been enriched and isolated in the right portion of panel G. The NG3 cells that were ChTx-/TnTx- were found to be 97.6% nestin+. The highest intensity nestin+ cells were found predominantly in the NG3 cells that were ChTx-/TnTx-.

Figure 3: Scanned photographs of (A) SVG EMEM in 10% FBS, (B) SVG NB + N2 and (C) NHNP NB +N2 as further described, for example, in Example 11.

## DETAILED DESCRIPTION

The present invention generally relates to immortalized multipotent human cell-lines derived from cells of the central nervous system, preferably derived from a fetal central nervous system, such as human fetal central nervous system, and methods of using these cell lines in treatment of disorders of the central nervous system. In particular, the cell lines and methods of the present invention may be used in the treatment of disorders caused by neurodegeneration in the central nervous system, such as Parkinsonism.

Moreover, the present invention provides methods of making the immortalized multipotent cells of the present invention as well as methods of identifying same. The cells, cell lines and cell types of the present invention are preferably at least partially isolated, isolated, and/or purified.

In one embodiment, the present invention provides methods of treating a host suffering from a central nervous system disorder, or alleviating the symptoms of such a disorder, by implanting immortalized human fetal cells derived from cells of the central nervous system. Preferably, the cells of the present invention will not produce graft rejection, intense intracerebral

inflammation, or tumor formation following implantation of such cells into the central nervous system. Further, the cells, after further differentiation, will preferably induce neuron migration and neurite extension, which will demonstrate that the cells are functioning to produce trophic factors that stimulate neuronal responses.

Implantation of immortalized multipotent human fetal cells derived from cells of the CNS, such as are provided by the present invention, will provide a means of treating many diseases. For example, Parkinson's disease will be treatable by implantation of these cells, or further differentiated cells derived from them, into the basal ganglia of an affected host. The trophic factors that should be produced by the differentiated cells derived from the implanted cells of the present invention, or implanted derived cells of the present invention, may inhibit dopaminergic neuron demise and even induce dopaminergic neuron regeneration or allow increased neurite outgrowth from existing neurons. The increased population of dopaminergic neurons can provide clinical improvement of persons suffering from Parkinsonism.

Moreover, as the multipotent cells of the present invention may develop into glial cells, it may be possible to provide regeneration of myelinating oligodendrocytes or multifunctional astrocytes.

In additional embodiments, the implanted cells may be transfected, *in vivo* or *in vitro*, with a nucleic acid that encodes a neurologically relevant polypeptide. The term "neurologically relevant peptide" generally refers to a peptide or protein that catalyzes a reaction within the tissues of the central nervous system. Such peptides may be naturally occurring neural peptides, proteins or enzymes, or may be peptide or protein fragments that have therapeutic activity within the central nervous system. Examples include neural growth factors, trophic factors, and cytokines, and enzymes used to catalyze the production of important neuro-chemicals, or their intermediates. In particularly preferred aspects, the cells will be transfected with a nucleic acid that encodes, for example, TH (tyrosine hydroxylase), GTPCH1 (GTP cyclohydrolase 1), AADC (aromatic amino acid decarboxylase), VMAT2 (vesicular monoamine transporter 2), GDNF (glial-derived neurotrophic factor), VEGF (vascular endothelial growth factor), BDNF (brain-derived neurotrophic factor), NGF (nerve growth factor), bFGF (also known as FGFII or basic fibroblast growth factor), CNTF (ciliary neurotrophic factor), PDGF (platelet-derived growth factor), BMP (which is known as a family of bone morphogenic proteins), LIF (Leukemia inhibitory factor), Neurturin, Persephin, Neublastin, NT4/5 (neurotrophin 4/5), NT3 (neurotrophin 3), Midkine, IL-10 (interleukin 10) or IL-6.

Tyrosine hydroxylase is the enzyme that converts tyrosine to L-DOPA, which is also the rate-limiting step in the production of dopamine. Therefore, expression of tyrosine hydroxylase by the implanted cells allows these cells to produce and secrete dopamine. Thus, in addition to promoting neuronal regeneration, the implanted cells may increase the dopamine concentration in the substantia nigra and limit or reverse the effect of dopaminergic neuron loss. When applied to the treatment of stroke, the peptide may aid in the revascularization of damaged nervous tissue or supply of neurotrophic factors that could enhance survival and regeneration of damage nervous tissue.

The methods of the present invention may also be used to treat other neurological disorders such as Huntington's chorea, epilepsy, stroke, Alzheimer's disease, traumatic brain injury, spinal chord injury, epilepsy, or multiple sclerosis. As immortalized human fetal CNS-derived cells of the present invention are expected to be compatible with the CNS, it should be possible to transfect these cells with DNA sequences encoding physiologically active peptides for implantation in the CNS, to effect treatment of other disorders. For instance, in Huntington's chorea and amyotrophic lateral sclerosis the peptide may block excitatory neurotransmitters such as glutamate. When applied to the treatment of multiple sclerosis, for example, the peptide would typically be a trophic stimulator of myelination, such as platelet derived growth factor or a ciliary neurotrophic factor which may block oligodendrocyte demise. As these diseases are more generalized than local lesions, alternative implantation methods may be desirable. For example, the cells may be implanted on a surface exposed to cerebrospinal fluid. Following expression and secretion, the peptide will be washed over the entire surface of the brain by the natural circulation of the cerebrospinal fluid. Suitable sites for implantation include the lateral ventricles, lumbar intrathecal region, and the like. In Alzheimer's disease, the cells may be transfected to produce nerve growth factor to support neurons of the basal forebrain as described by Rosenberg et al., Science, 242:1575-1578 (1988), incorporated herein by reference.

The cell lines and cell types of the present invention may therefore serve as gene vectors.

The methods of the present invention may also be employed to treat hosts by implantation of cells in extraneural sites. This embodiment of the present invention is particularly useful for prophylactic treatment of a host. Immortalized multipotent human fetal neuro-derived cells may be transfected with DNA encoding a disease-associated antigen, e.g., HIV gp120 polypeptides that encompass the principal neutralizing domain of HIV as described, e.g., in U.S. Pat. No. 5,166,050. The cells may then express and secrete the antigen encoded by the transfected DNA.

The antigen may be continuously secreted by the implanted cells and elicit a strong immune response. Following an adequate time interval to fully immunize the host, the cells may be removed.

As used herein, "treating a host" includes prophylactic, palliative, and curative intervention in a disease process. Thus, the term "treatment" as used herein, typically refers to therapeutic methods for reducing or eliminating the symptoms of the particular disorder for which treatment is sought. The term "host," as used herein, generally refers to any warm blooded mammal, such as humans, non-human primates, rodents, and the like, which is to be the recipient of the particular treatment. Typically, the terms "host" and "patient" are used interchangeably herein to refer to a human subject.

A wide variety of diseases and syndromes may be treated by the methods of the present invention. Generally, the disease will be a neurological disease including, but not limited to Parkinsonism (including Parkinson's disease), Alzheimer's disease, epilepsy, Huntington's chorea, multiple sclerosis, amyotrophic lateral sclerosis, Gaucher's disease, Tay-Sachs disease, neuropathies, brain tumors, stroke. The methods of the present invention may also be employed in the treatment of non-neurological diseases. For example, the methods of the present invention may be used to immunize hosts against infectious diseases, such as viruses, bacteria, protozoa, and the like as described above. Immortalized multipotent human fetal neuro-derived cells of the present invention may be transfected by DNA encoding physiologically active peptides or peptides which contain immunological epitopes. The methods of the present invention may be employed to implant the peptide producing cells and provide continuous *in vivo* delivery of other types of peptides, such as growth hormone, to the host.

In order to practice the methods of treatment described above, the present invention also provides cell lines suitable for transplantation into a host or patient.

In general, the cells implanted by the methods of the present invention are immortalized multipotent human fetal CNS-derived cells. By "neuro-derived" or "CNS-derived", it is meant that, prior to immortalization, the cells were harvested from the CNS and/or had a neurological cell phenotype (neuronal or glial). Neurological cell types include neurons, astrocytes, oligodendrocytes, choroid plexus epithelial cells, and the like.

Cells of the CNS have been categorized as developing from stem cells into neuronal cells and glial cells . McKay, Science, 276: 66-71 (1997). Along the path from stem cell to either neuronal cell or glial cells, a stem cell in the CNS has the ability to become a neuroblast

(immature neuron) or a glioblast (immature glia) before further differentiating into mature neuronal cells (neurons) and glial cells (astrocytes and oligodendrocytes). It is generally believed that multipotent CNS derived stem cells require mitogenic factors, such as basic fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF), to be propagated in culture . Flax *et al.*, Nat Biotechnol, 16: 1033-9 (1998); Gage *et al.*, Annu Rev Neurosci, 18: 159-92 (1995); Kitchens *et al.*, J Neurobiol, 25: 797-807 (1994); Kuhn *et al.*, J Neurosci, 17: 5820-9 (1997); Morshead *et al.*, Neuron, 13: 1071-82 (1994); Palmer *et al.*, Mol Cell Neurosci, 8: 389-404 (1997); Reynolds *et al.*, J Neurosci, 12: 4565-74 (1992); Sah *et al.*, Nat Biotechnol, 15: 574-80 (1997); Shihabuddin *et al.*, Exp Neurol, 148: 577-86 (1997); Weiss *et al.*, J Neurosci, 16: 7599-609 (1996); Zhou & Chiang, Wound Repair Regen, 6: 337-48 (1998). In practice, stem cells are passaged in serum-free media culture . Fisher, Neurobiol Dis, 4: 1-22 (1997); Flax *et al.*, Nat Biotechnol, 16: 1033-9 (1998); Gage *et al.*, Annu Rev Neurosci, 18: 159-92 (1995); Kitchens *et al.*, J Neurobiol, 25: 797-807 (1994); Kuhn *et al.*, J Neurosci, 17: 5820-9 (1997); Mokry *et al.*, Sb Ved Pr Lek Fak Karlovy Univerzity Hradci Kralove, 38: 167-74 (1995); Morshead *et al.*, Neuron, 13: 1071-82 (1994); Palmer *et al* ., Mol Cell Neurosci, 8: 389-404 (1997); Sah *et al.*, Nat Biotechnol, 15: 574-80 (1997); Shihabuddin *et al.*, Exp Neurol, 148: 577-86 (1997); Weiss *et al.*, J Neurosci, 16: 7599-609 (1996); Zhou & Chiang, Wound Repair Regen, 6: 337-48 (1998) because, the addition of serum and removal of the mitogenic factor(s) has been found to result in the differentiation of the stem cells (Reynolds, 1992; Stemple, 1992; Anderson 92; Flax, 1998) into either neuronal or glial cell types.

A CNS derived stem cell is defined as a cell capable of differentiating into neurons, astrocytes or oligodendrocytes and self-renewing sufficiently to populate the brain . McKay, Science, 276: 66-71 (1997). A progenitor cell has a more restricted potential cellular diversity than a stem cell, but may still form neurons or glia. A precursor cell has an even more restricted phenotype, such as a neuroblast, that can only become a neuron. One of ordinary skill in the art will appreciate however that the development of cells from multipotent to differentiated cell types may be defined by the appearance and disappearance of a number of histological markers, some of which are described herein. While the terms stem cells, progenitor cells and precursor cells are used herein, these terms should not limit the characterization of the multipotent immortalized cells described herein and are not meant to describe terminal steps along the pathway to complete differentiation. That is, for example, it is possible that progenitor cells may be capable of exhibiting characteristics of stem cells, depending on environmental conditions,

such as culture media. These are relative descriptors on a relative scale of specialization with each differentiating step leading to further restrictions of potential cellular phenotypes.

A multipotent cell therefore, as referred to herein is a cell with the potential to express multiple phenotypes and includes stem, progenitor and precursor cells.

Identification of stem cells in the CNS has routinely been performed using an antibody to intermediate filament protein nestin . Lendahl *et al.*, Cell, 60: 585-95 (1990); Reynolds *et al.*, J Neurosci, 12: 4565-74 (1992). Nestin is expressed in proliferating CNS stem/progenitor cells . Frederiksen & McKay, J Neurosci, 8: 1144-51 (1988); Reynolds *et al.*, J Neurosci, 12: 4565-74 (1992); Stemple & Anderson, Cell, 71: 973-85 (1992); Vescovi *et al.*, Neuron, 11: 951-66 (1993). Another marker that has been used to identify neural progenitor cells is the intermediate filament protein vimentin . Flax *et al.*, Nat Biotechnol, 16: 1033-9 (1998; Kilpatrick & Bartlett, Neuron, 10: 255-265 (1993).

There is a growing appreciation that there is tremendous plasticity of stem cells as demonstrated by recent demonstrations that CNS derived stem cells may be used to form differentiated hematopoietic cells . Bjornson *et al.*, Science, 283: 534-7 (1999). Similarly, stromal marrow stem cells have been used to create cells of the brain, including neurons and astrocytes . Azizi *et al.*, Proc Natl Acad Sci U S A, 95: 3908-13 (1998); Pereira *et al.*, Proc Natl Acad Sci U S A, 95: 1142-7 (1998); Prockop, J Cell Biochem Suppl, 31: 284-5 (1998); Prockop, Science, 276: 71-4 (1997); Sanchez-Ramos J., Movement Disorders, 13: 122(p2.149) (1998). Therefore, it may be possible to make a broader diversity of cell types from CNS derived stem cells, such as are disclosed herein, than neurons and glia. In fact, nestin expression has also been found in myoblasts, which are muscle precursor cells, which underscores the plasticity of cells that express nestin . Kachinsky *et al.*, Dev Biol, 165: 216-28 (1994).

It will be appreciated that as the present invention provides multipotent immortalized cells, it may be possible to also treat diseases associated with or requiring hematopoietic cell replacement, such as a part of chemo- or radiation therapy. It may be possible for the presently provided multipotent immortalized cells to repopulate bone marrow cells and differentiate *in vivo* or be differentiated *in vivo* or *in vitro* to produce hematopoietic cells.

Once the possibility of using stem cells or multipotent cells for transplantation therapy, such as is described herein, is appreciated, the practical difficulty remained that there is a limited supply of these cells and their identification, isolation and expansion from natural sources is difficult. While there have been reports of immortalizing genes being introduced into stem cells,

there have been, to date, no reports of truly immortalized stem or multipotent cells derived from the CNS. Immortalized cell lines are now recognized as a limitless supply of cells that may be obtained from a post-crisis (post-senescence) parental population. Immortalization of a cell line usually requires passing cells usually containing an immortalizing gene through the period of senescence. While human CNS stem/progenitor cells have been previously transfected to contain an immortalizing gene which encodes the v-myc protein, Flax *et al.*, Nat Biotechnol, 16: 1033-9 (1998; Sah *et al.*, Nat Biotechnol, 15: 574-80 (1997), it has not been shown that these transfected CNS progenitors can be propagated in culture indefinitely (i.e. > 75, preferably greater 85, more preferably greater than 100 passages) or that they have passed through crisis to become immortalized.

Preparation of the immortalized multipotent fetal cell lines may generally be carried out according to the following procedures. Fetal cells may be collected following elective abortion. Women donating fetuses following abortion will typically be serologically screened for a variety of infectious diseases, including human immunodeficiency virus, hepatitis B virus, hepatitis C virus, cytomegalovirus, and herpes viruses Types 1 and 2.

The fetal brain is identified and collected. The cells may be prepared as follows: brain tissue is aspirated through a 19 gauge needle and washed twice in Eagle's minimum essential media (EMEM, Gibco, New York, N.Y.). Cells are plated on culture dishes treated with poly-D-lysine (0.1 mg/ml for 5 minutes). The cells are grown on EMEM supplemented with 20% fetal bovine serum, 75 g/ml streptomycin, 75 units/ml penicillin, 1% dextrose and 2 g/ml fungizone (Gibco). Prior to immortalization the cells are incubated at 37°C in a 5% $CO_2$ humidified environment. One of skill in the art will recognize that other methods for preparing cells may also be used. For example, the tissue source for obtaining these populations of cells can be nonfetal (Takahashi, J., Palmer, T. D., Gage, F. H. "Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures". J. Neurobiol 38:65-81, 1999).

The cells to be implanted by the methods of the present invention can be immortalized by a variety of techniques. Typically, the cells will be immortalized by introduction of an immortalizing gene followed by passage of the cells through crisis. This cell population is referred to herein as a NG1 population of cells. The original cell cultures that contain an immortalizing gene, but have not passed through crisis, are expected to contain and produce multipotent, stem and/or progenitor cells, neuronal and glial cells. It should be possible to purify

these progenitor or multipotent cells from this original cell culture, with the methods described herein, prior to immortalization or even possibly prior to introduction of the immortalizing gene. A preferred method, and the method exemplified herein however, involves introduction of an immortalizing gene, culturing the population of CNS derived fetal cells containing the immortalizing gene through crisis to thereby obtaining a more highly enriched population of multipotent, stem or progenitor immortalized CNS derived cells.

Prior to introduction of the immortalizing gene, the CNS derived fetal cells will survive for several months with regular re-feeding, but show little cell proliferation. The CNS derived fetal cells are preferably transfected with a replication incompetent SV40 deletion mutant, such as is described in Major (U.S. Patent No. 4,707,448). However, a truncated SV40 can be used as an immortalizing gene . Truckenmiller *et al.*, Cell Tissue Res, 291: 175-89 (1998).

While the presently exemplified method of making the cells of the present invention involves introduction of an SV40 gene, the cells may alternatively be immortalized by other techniques that are well known in the art. For example, immortalization by Epstein-Barr virus may be employed, as described in U.S. Pat. No. 4,464,465, incorporated herein by reference. Epstein-Barr virus mutants which lack OriP and OriLyt origins of replication are particularly useful. Another useful method of immortalization is over-expression of a cellular gene for growth control such as c-myc as described by Bartlett et al., Proc. Natl. Acad. Sci. USA, 85:3255-3259 (1988), incorporated herein by reference.

Generally, cells suitable for further immortalization procedures according to the present invention will be anchorage dependent, will not grow in soft agar, and will not exhibit focus formation. The cells will also have a generation time equal to normal human cells in culture and be contact inhibited for growth.

In one embodiment, the present invention provides a population of immortalized multipotent CNS derived fetal cells that are generally referred to herein as a NG1 population. The SVG cell line deposited with the American Type Culture Collection, Manassas, VA (A.T.C.C. CRL 8621) which is described in U.S. Pat. No. 4,707,448, incorporated herein by reference, is particularly useful as a starting material to make the NG1 cell population of the present invention. Hereinafter by "SVG cells" or "SVG cell line", it is meant cells or a cell line derived from cell line A.T.C.C. CRL 8621. By derivatives is meant a subclone, replication, or genetically altered mutant of cell line A.T.C.C. CRL 8621.

The heterogeneous pre-crisis human cell line was established by methods provided in patent 4,707,448 and Major *et al.*, Proc Natl Acad Sci U S A, 82: 1257-61 (1985) by using a replication incompetent ,.Gluzman, Cell, 23: 175-82 (1981), origin-defective-mutant (ori$^-$) of SV40 virus to express the SV40 immortalizing gene in human fetal multipotent cells. These cells, which were designated SVG, provide rapidly growing cultures containing human astroglial cells which are capable of reproducing infectious JC virus following infection or transfection in concentrations and at the same rate as primary human fetal glial cells.

In a preferred embodiment of the invention, SVG cells are propagated in standard media and passed through the crisis period to be immortalized. Only a small fraction of the heterogeneous parental SVG cells survived crisis which is typical for SV40 immortalization . Bryan & Reddel, Crit Rev Oncog, 5: 331-57 (1994) . Those SVG cells that emerged from crisis differed from the parental SVG cells in that they were growth immortalized and thus are considered a distinct cell population termed herein as NG1. The onset of crisis has been found to begin at approximately 33-38 passages of the SVG cells. During crisis, there is very little cell growth and the cells look very large and granular. When SVG cells were in crisis, there was observable cell death when cells are viewed under the microscope. When cells in crisis are fed twice weekly and kept at a cell density $\geq$ 1.5x106/T75 flask, a rare colony of cells sometimes will emerge from crisis and begin growing. The generation of a post-crisis SV40 immortalized human CNS derived cell line and the cell line itself (as well as the cell types contained therein) are unique and potentially useful for therapeutics and the development of therapeutics, as described herein.

According to this embodiment, SVG cells were grown in "standard medium" including Eagle Minimum Essential Medium (EMEM; BioWhittaker Cat# 12-125F) + 10% FBS (BioWhittaker Cat# 14-501F) + 2mM l-glutamine (Quality Biological, Gaithersburg, MD). Passaging of the cells was accomplished by removal of the media and washing 2x with Hanks' balanced salt solution (HBSS: BioWhittaker, Walkersville, MD), 3 minute incubation with 0.25% trypsin-EDTA solution (BioWhittaker) to dissociate the cells. The suspended cells were added to fresh standard media and centrifuged at 100x g at room temperature for 5 minutes. The cell pellet was then brought up in fresh standard media and plated in a new 75 cm2 flask.

Pre-crisis SVG cells were subcultured every 3-4 days when the flask was confluent, but during crisis they only needed to be subcultured every 8-10 days due to slow growth and cell death. While in crisis, the SVG cells did not need to be subcultured as frequently, but continued

to be seeded at 1.5x106 per 75 cm2 flask. The SVG cells were able to remain in crisis for about 2 months before one or more colonies of cells emerged and began growing in the flask. These colonies, which were mixed together with each passage, outgrew the other cells in the flask and constituted the post crisis cell line. Useful post-crisis cell types may be obtained after approximately 38 passages, however, useful post crisis multipotential cells may also be obtained, for example, after 38-50 passages, such as, for example 44, 45, 47 or 49 passages. The NG1 post-crisis cell line was generated by seeding SVG cells into a designated flask and treating it as a separate cell line as it went through and emerged from crisis. The NG1 cell line is not considered clonal, since it represents a pool of all the colonies that emerged from crisis within the flask.

Further embodiments of the present invention are based on the unexpected discovery that fetal CNS-derived cells which contain an immortalizing gene, such as those contained within the SVG cell population, express a variety of cell markers. It has now been found that the relative expression of phenotypic markers for neurons or glial cells, and the differentiation of the multipotent CNS-derived cells within the NG1 population of the present invention as well as the original pre-crisis (such as SVG) population, can be intentionally modulated by changes in the media composition. Accordingly, the present invention provides methods of enriching mixed populations of CNS-derived fetal cells for different cell types. Specifically, it has been unexpectedly found that the removal of serum from the culture conditions enriches the population of cells with a greater number of cells identified as cells which are expected to differentiate to a neuronal lineage. While this effect has been demonstrated for the post-crisis, NG1 cell population, it is also expected to be equally applicable to the pre-crisis original cells (such as the SVG population). The enriched cell population produced from the SVG population is referred to herein as a NG2 population and the enriched population produced from the post-crisis NG1 population is referred to herein as a NG3 population. Representative identification of the cell types contained in these populations are described in the following however one of ordinary skill will appreciate that these exemplifications for the populations are not limiting.

The possible effects of agents on the differentiation of the parental pre-crisis (and post-crisis) SVG cells was first observed by the extension of neurite-like processes upon addition of 12-myrstate 13 acetate (PMA) to the standard media. This ability has been further confirmed by the recent report that addition of forskolin, which elevates cAMP, can increase gap junctional communication and expression of glial fibrillary acidic protein (GFAP; a differentiated glial

marker) and neurofilament (NF; a differentiated neuronal marker) . Dowling-Warriner & Trosko, FASEB J. Abstr., 4382 (1998) and Dowling-Warriner & Trosko, Neuroscience 95:859-868 (2000). This ability to control differentiation and the distinctive cellular phenotypes suggested the possibility to create new cell populations and/or alter the proportion of cell types within a cell population. The ability to alter the types of cells within the population, and specifically to enrich for a greater number of multipotent cells and neural precursor cells from a population of fetal CNS-derived cells that have been passaged in serum is particularly unexpected. It is generally thought that in the absence of exogenous mitogens and the presence of serum, neurons and astrocytes would have been expected to have already differentiated . Palmer *et al.*, Mol Cell Neurosci, 8: 389-404 (1997; Sah *et al.*, Nat Biotechnol, 15: 574-80 (1997).

In another embodiment, the present invention provides methods of identifying and purifying the multipotent fetal CNS-derived cells described herein, as well as the specific cell types.

One of the major difficulties encountered when studying the development of the CNS has been the inability to readily identify specific cell lineages at distinct phases of proliferation and differentiation due, in part, to the lack uniquely specific cell markers. There is at present a rapidly growing number of commercially available polyclonal and monoclonal antibodies which can be used to detect specific cell surface, cytoplasmic or nuclear epitopes in CNS cells. However, many of these epitopes are shared among neuronal and glial cell types at some stages of their development. Therefore, there is an increasing need for using double- and triple-immunolabeling procedures using fluorescent probes, in order to obtain a more precise identification of specific cell populations under investigation. A flow cytometer equipped with dual and triple emission filter sets to detect fluorescence is ideally suited to access this complexity and diversity of specific CNS populations in a very rapid and precise manner. While the preferred method of the present invention involves the use of a flow cytometer, other methods of identifying and sorting cells including panning and magnetic beads may be used.

In the present invention, three cellular markers have been used to characterize the specific cell types of the invention. These cell markers include tetanus toxin fragment C (TnTx), cholera toxin (ChTx) and monoclonal antibodies designated A2B5. Tetanus toxin fragment C (TnTx) receptor is a marker of terminally post-mitotic developing neurons (Koulakoff *et al.*, Dev Biol, 100: 350-7 (1983)) that express the tetanus toxin receptor. There are a number of antibodies designated as A2B5. These antibodies recognize a mixture of sialic acid residues on the cell

surface of specific cell populations. Due to the heterogeneity of the sialic acid residues with which the A2B5 antibodies bind, the designation for A2B5 positive (A2B5+) cells indicated those cells that bind with the A2B5 antibody.

A2B5 antibodies are used as a neuronal and glial progenitor cell marker . Abney *et al.*, Dev Biol, 100: 166-71 (1983; Fredman *et al.*, Arch Biochem Biophys, 233: 661-6 (1984; Rao & Mayer-Proschel, Dev Biol, 188: 48-63 (1997). Cholera toxin (Cholera toxin-FITC, Sigma, St. Louis, MO) is used to label undifferentiated neurons . Shindler & Roth, Brin Res Dev Brain Res, 92: 199-210 (1996) or progenitor cells.

Neural stem/progenitor cells were identified by the expression of the intermediate filament protein nestin . Lendahl *et al.*, Cell, 60: 585-95 (1990). Identification of stem cells from the CNS has routinely been performed using an antibody to intermediate filament protein nestin. Lendahl *et al.*, Cell, 60: 585-95 (1990); Reynolds *et al.*, J Neurosci, 12: 4565-74 (1992). Nestin has been found to also be expressed in proliferating neural stem/progenitor cells . Frederiksen & McKay, J Neurosci, 8: 1144-51 (1988); Reynolds *et al.*, J Neurosci, 12: 4565-74 (1992); Stemple & Anderson, Cell, 71: 973-85 (1992; Vescovi *et al.*, Neuron, 11: 951-66 (1993).

The antibody to nestin used in the present experiments is a rabbit polyclonal immunoglobulin designated as nestin-331B identifies human nestin (Messam *et al.*, Exp Neurol., 161:585-596 (2000)). The antibody to human nestin has been characterized to be a marker for CNS stem/progenitor cells that have the potential to become neurons or glia (Messam, *et al.*, Exp Neurol 161:585-596, 2000).

Antibodies to nestin are available from Boehringer Mannheim Biochemicals, Indianapolis, IN (antibody to rat nestin) or an antibody that recognizes human nestin may be requested from R. McKay (NIH, Bethesda, MD). The human nestin gene sequence has been cloned . Dahlstrand *et al.*, J Cell Sci, 103: 589-97 (1992) and is available through publicly available databases such that antibodies to human nestin may be made by means known in the art. For example, with a known nestin sequence, it is straightforward to use an epitope mapping program algorithm to obtain a highly antigenic peptide fragment. Thus, 6 up to >50 amino acid length peptide can be synthesized by commercial vendors (Peptides International, Louisville, KY) and conjugated to KLH or another carrier molecule to enhance immunogenicity of the peptide. Adequate amounts > 1 mg / inoculation can be given to rabbits subcutaneously and 3 booster inoculations may be provided typically at 4, 8, 12-16 weeks. Methods previously have been described for the efficient production of polyclonal antibodies or monoclonal antibodies

that could be used in this application. Jennes & Stumpf, Neuroendocrine Peptide Methodology, 42: 665 (1989; Youngblood & Kizer, Neuroendocrine Peptide Methodology, 38: 605 (1989).

A further indication of the differentiation lineage of the cell types of the present invention was provided by the ability of JC virus to infect any given cell type. More specifically, one of ordinary skill will appreciate that JC virus preferentially infects glial cells, as opposed to neuronal cells. JC virus more preferentially infects oligodendrocytes, as opposed to astrocytes . Major *et al.*, Clin Microbiol Rev, 5: 49-73 (1992).

Another marker that has been used to identify neural progenitor cells, and could be used to further characterize the cell types of the present invention, is the intermediate filament protein, vimentin . Flax *et al.*, Nat Biotechnol, 16: 1033-9 (1998; Kilpatrick & Bartlett, Neuron, 10: 255-265 (1993). Consistent with the multipotent nature of the NG1 cells, these cells also express vimentin . Tornatore *et al.*, Cell Transplant, 5: 145-63 (1996).

The ability of the method of the present invention to enrich populations of cells for multipotent cell types as well as a characterization of exemplary cell populations are shown in Table 1 with data summarized from Figures 1-3.

**Table 1**: NG1 and NG3 cell populations, as derived from SVG population

| **Phenotype** | **EMEM + 10% FBS (NG1 population)** | **Neurobasal + N2 supplements (NG3 population)** |
|---|---|---|
| ChTx+ | 97% | 99% |
| ChTx+, TnTx+ | 2% | 18% |
| A2B5+ | 23% | 7% |
| ChTx -, TnTx - | | 97% are nestin+ |
| JC virus sensitive | 35-40% | 0% |

ChTx+: Cholera toxin

TnTx+: Tetanus toxin

A2B5+: glial or neuronal progenitor

JC virus infects cells of glial lineage

The 14 specific cell types (type number indicated in parentheses) of the present invention are defined as follows with regard to their expression of the markers described above that could arise from NG1 or NG3 cell populations.

TnTx-/ChTx- (1)

TnTx-/ChTx+ (2)

TnTx+/ChTx- (3)

TnTx+/ChTx+ (4)

A2B5-/TnTx- (5)

A2B5+/TnTx+ (6)

A2B5+/TnTx- (7)

A2B5-/TnTx+ (8)

A2B5-/ChTx- (9)

A2B5+/ChTx+ (10)

A2B5-/ChTx+ (11)

A2B5+/ChTx- (12)

Type 1 cells above can be further divided into nestin+ (Type 13) and nestin- (Type 14) cells that are also TnTx-/ChTx-.

Of these, types (1, 5 and 9) are expected to be the most immature since they are negative for the phenotypic markers. The TnTx-/ChTx- type 1 cells in Neurobasal medium + N2 supplements have about 1% A2B5+ cells in the total population (Figure 3). When these type 1 cells are gated out, 95% of the remaining cells are nestin+. This nestin+/TnTx-/ChTx- cell phenotype likely represents stem/progenitor cells that have not entered into either a glial or neuronal pathway. These cells have characteristics consistent with stem/progenitor cells that may have the potential to differentiate into types of neurons or different types of glial cells i.e. astrocytes or oligodendrocytes.

Other cell types of the present invention are identified using a combination of markers. The following is a description regarding the significance of the phenotypes that is not intended to be limiting to the final neuronal or glial phenotype of the cells.

A ChTx+/TnTx- (cell-type 2 or possibly 11) cell phenotype is generally considered a pre-neuronal marker. Whereas ChTx+/TnTx+ (type 4 cells) identifies cells as post mitotic neurons.

These type 4 cells predominantly go along a neuronal pathway, particularly if instructed in the presence of factors which potentiates neuronal growth.

A2B5 positive cells can be either neurons or glial cells. ChTx+ and/or TnTx+ (cell types 2-4) identifies cells as likely to be neurons. A2B5+/ChTx- or A2B5+/TnTx- cells (cell types 7 or 12) are likely in a glial lineage unless instructed to become neuronal. A2B5+/ChTx+ cells (cell type 10) can differentiate into either glial or neuronal pathways. A2B5-/TnTx+ (cell type 8) identifies cells as likely to be neurons.

The NG1 cells maintained on EMEM 10% sustains cells that are phenotypically neuronal or glial, but when placed into a neuron environment i.e. Neurobasal + N2 supplements, will preferentially develop into neurons. There were also the TnTx-/ChTx- type 1 cells that were nestin+. This population is enhanced in the Neurobasal + N2 supplements condition. The shift in phenotypes in these cultures, as instructed by metabolic conditions or factors in the medium, indicates that the NG1 cells in culture are maintained as a heterogeneous population of cells with precursor properties able to differentiate by specific cues into either more mature glial or neuronal pathways.

The present invention is based, in part, on the discovery that the cells of the populations described herein are not a single phenotype and that multiple phenotypes can arise by intentional alteration ('instruction') of the environment or culture of the populations.

A method of the present invention includes analysis of the phenotypes of the cells of these populations with at least 3 markers, preferably in combination, to define preferred phenotypes of cells of the invention (Figure 2).

Beyond providing the specific cell types described above, the present invention provides a composition containing one or more of these cell types, preferably in a pharmaceutically acceptable excipient.

These markers have also been useful in demonstrating the method of enrichment of cell populations, which is a further embodiment of the present invention. Specifically, as further detailed in the examples, a NG1 population of cells was characterized as shown in Table 2 as a function of the culture conditions.

These results demonstrate the enrichment of already existing and/or formation of a greater number of multipotent and immature neural cell types by the method of the present invention.

The cells of the present invention, either cell populations or enriched cell types, may be prepared for implantation by suspending the cells in a physiologically compatible carrier, such as cell culture medium (e.g., Eagle's minimal essential media) or phosphate buffered saline. Cell density may generally be about $10^4$ to $10^7$ cells/ml. The cell suspension is gently rocked prior to implantation. The volume of cell suspension to be implanted will vary depending on the site of implantation, treatment goal, and cell density in the solution. Typically, the amount of cells transplanted into the patient or host will be a "therapeutically effective amount." As used herein, a therapeutically effective amount refers to the number of transplanted cells which are required to effect treatment of the particular disorder for which treatment is sought. For example, where the treatment is for Parkinsonism, transplantation of a therapeutically effective amount of cells will typically produce a reduction in the amount and/or severity of the symptoms associated with that disorder, e.g., rigidity, akinesia and gait disorder. In the treatment of Parkinsonism, the amount of cells to be administered in each injection will be sufficient to achieve this effective amount. Several injections may be used in each host. Persons of skill will understand how to determine proper cell dosages.

As noted above, it may be possible to transplant the cell types or enriched cell populations of the present invention directly or, alternatively, it may be preferable to further differentiate the cell types or populations further prior to transplantation.

In alternative preferred embodiments of the present invention, the cells that are useful for transplantation, may be transfected with, and capable of expressing, a heterologous nucleic acid sequence which encodes a neurologically relevant peptide. The term "heterologous" as used to describe the nucleic acids herein, generally refers to a sequence which, as a whole, is not naturally occurring within the cell line transfected with that sequence. Thus, the heterologous sequence may comprise a segment which is entirely foreign to the cell line, or alternatively, may comprise a native segment which is incorporated within the cell line in a non-native fashion, e.g., linked to a non-native promoter/enhancer sequence, linked to a native promoter which is not typically associated with the segment, or provided in multiple copies where the cell line normally provides one or no copies.

Generally, the nucleic acid sequence will be operably linked to a transcriptional promoter and a transcriptional terminator. A DNA segment is operably linked when it is placed into a functional relationship with another DNA segment. For example, a promoter or enhancer is operably linked to a coding sequence if it stimulates the transcription of the sequence; DNA for a

signal sequence is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide. Generally, DNA sequences that are operably linked are contiguous, and in the case of a signal sequence both contiguous and in reading phase. However, enhancers need not be contiguous with the coding sequences whose transcription they control. Linking is accomplished by ligation at convenient restriction sites or at adapters or linkers inserted in lieu thereof. The DNA sequence may also be linked to a transcriptional enhancer. Expression of the DNA in the implanted cells may be constitutive or inducible. A variety of expression vectors having these characteristics may carry the DNA for transfection of the cells, such as plasmid vectors pTK2, pHyg, and pRSVneo, simian virus 40 vectors, bovine papillomavirus vectors or Epstein-Barr virus vectors, as described in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, 1988. These vectors are may also include the vectors pcDNA3.1 and EF-1 that are commercially available (Invitrogen, Carlsbad, CA) or previously incorporated herein by reference. The vectors may be introduced into the cells by standard methods, such as electroporation, calcium phosphate-mediated transfection, polybrene transfection, cationic lipids . Felgner & Gadek, Proc Natl Acad Sci USA, 84: 7413 (1987) and the like, as described in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, 1988.

In addition, the present invention contemplates the use of promoter systems that can be regulated by the exogenous addition of compounds. These regulatable promoter systems allow some control of the dose of the encoded peptide/protein. Four separate leading technologies currently exist and have been developed for regulating the expression of target genes *in vitro* and *in vivo*. These include (1) the tetracycline based gene switch, (2) the RU486 based gene switch, (3) the ecdysone based gene switch and (4) the FK1012 (rapamycin) based gene switch.

The peptide encoded by the nucleic acid may generally be a directly therapeutic compound, such as a movement inhibitor in the treatment of Huntington's chorea. Alternatively, the peptide encoded by the nucleic acid may be selected to supplement or replace deficient production of the peptide by the endogenous tissues of the host, which deficiency is a cause of the symptoms of a particular disorder. In this case, the cells or cell populations act as an artificial source of the peptide. Alternatively, the peptide may be an enzyme that catalyzes the production of a therapeutic, or neurologically relevant compounds. Again, such compounds may be exogenous to the host's system, or may be an endogenous compound whose synthesis pathway is otherwise impaired. In this latter case, production of the peptide within the CNS of the host

provides supplemental pathways for the production of the compound. For example, in a preferred embodiment, the immortalized human fetal neuro-derived cell lines are transfected with a nucleic acid that encodes a tyrosine hydroxylase enzyme. Tyrosine hydroxylase catalyzes the synthesis of L-dopa from tyrosine. Dopamine has been demonstrated to be effective in the treatment of Parkinsonism.

The nucleic acid may also encode a trophic factor such as a nerve growth factor, an inhibitory growth factor, or a cytokine useful in the treatment of brain tumors. Due to their ability to enhance neural regeneration and produce and secrete L-dopa, the cells and cell populations of the present invention are particularly useful in the treatment of central nervous system disorders which are associated with the loss of dopaminergic cells in the CNS of the host, such as Parkinsonism.

Typically, the cells or cell populations of the present invention may be implanted within the parenchyma of the brain, in a space containing cerebrospinal fluid, such as the sub-arachnoid space or ventricles, or extraneurally. As used herein, the term "extraneurally" is intended to indicate regions of the host which are not within the central nervous system or peripheral nervous tissue, such as the celiac ganglion or sciatic nerve. "Extraneural" regions may contain peripheral nerves. "Central nervous system" is meant to include all structures within the dura mater.

When the cells or cell populations of the present invention are implanted into the brain, stereotaxic methods will generally be used as described in Leksell and Jernberg, Acta Neurochir., 52:1-7 (1980) and Leksell et al., J. Neurosurg., 66:626-629 (1987), both of which are incorporated herein by reference. Localization of target regions will generally include pre-implantation MRI as described in Leksell et al., J. Neurol. Neurosurg. Psychiatry, 48:14-18 (1985), incorporated herein by reference. Target coordinates will be determined from the pre-implantation MRI.

Prior to implantation, the viability of the cells may be assessed as described by Brundin et al., Brain Res., 331:251-259 (1985), incorporated herein by reference. Briefly, sample aliquots of the cell suspension are mixed on a glass slide with of a mixture of acridine orange and ethidium bromide at the prescribed ratio of each component in 0.9% saline; Sigma). The suspension is transferred to a hemocytometer, and viable and non-viable cells were visually counted using a fluorescence microscope under epi-illumination at 390 nm. combined with white light trans-illumination to visualize the counting chamber grid. Acridine orange labels live nuclei green, whereas ethidium bromide will enter dead cells resulting in orange-red fluorescence. Cell

suspensions should generally contain more than about 50%, preferably 75%, more preferably 85%, alternatively 95%, viable cells.

Injections will generally be made with sterilized 10 :l Hamilton syringes having 23-27 gauge needles. The syringe, loaded with cells, are mounted directly into the head of a stereotaxic frame. The injection needle is lowered to predetermined coordinates through small burr holes in the cranium, 40-50 :l of suspension are deposited at the rate of about 1-2 :l/min. and a further 2-5 min. are allowed for diffusion prior to slow retraction of the needle. Frequently, at least two separate deposits will be made, separated by 1-3 mm, along the same needle penetration, and up to 5 deposits scattered over the target area can readily be made in the same operation. The injection may be performed manually or by an infusion pump. At the completion of surgery following retraction of the needle, the host is removed from the frame and the wound is sutured. Prophylactic antibiotics or immunosuppressive therapy may be administered as needed.

For treatment of more generalized neurological disorders, cells may be transfected to express a therapeutic compound and implanted in the ventricles or lumbar theca. As the therapeutic compound is secreted by the cells, natural circulation of the cerebrospinal fluid washes the therapeutic compound throughout the central nervous system providing a means of generalized treatment. Implantation into the ventricles may be accomplished by an open procedure, such as described in Madrazo et al., New Engl. J. Med., 316:831-834 (1987) or Penn et al., Neurosurgery, 22:999-1004 (1988), both of which are incorporated herein by reference. Implantation of cells into the lumbar theca is most conveniently accomplished by standard procedures similar to instillation of radiographic contrast media or anti-tumor medication via a lumbar puncture.

In some instances, it may be desirable to implant cells extraneurally according to the present invention. The cells may be implanted percutaneously through a needle or endoscope or by an open procedure. Persons of skill will readily appreciate the most appropriate method of implanting cells for particular applications.

The cells or cell populations may be encapsulated by membranes prior to implantation. The encapsulation provides a barrier to the host's immune system and inhibits graft rejection and inflammation. Several methods of cell encapsulation may be employed. In some instances, cells will be individually encapsulated. In other instances, many cells will be encapsulated within the same membrane. When the cells will be removed following implantation, the relatively large size of a structure encapsulating many cells within a single membrane provides a convenient means

for retrieval of the implanted cells. Several methods of cell encapsulation are well known in the art, such as described in European Patent Publication No. 301,777, or U.S. Pat. Nos. 4,353,888, 4,744,933, 4,749,620, 4,814,274, 5,084,350, or 5,089,272, each of which is incorporated herein by reference.

One method of cell encapsulation is as follows. The cells are mixed with sodium alginate (a polyanionic seaweed extract) and extruded into a solution of divalent cations, e.g., calcium chloride, which complexes with the sodium alginate to form a gel, resulting in the formation of gelled beads or droplets that contain the cells. The gel beads are incubated with a high molecular weight (MW 60-500x $10^3$) concentration (0.03-0.1% w/v) polyamino acid, such as poly-L-lysine, for a brief period of time (3-20 minutes) to form a membrane. The interior of the formed capsule is reliquified by treating with sodium citrate. The single membrane around the cells is highly permeable (MW cut-off 200-400x $10^3$). The single membrane capsule containing the cell is incubated in a saline solution for 1-3 hours to allow entrapped sodium alginate to diffuse out of the capsule and expand the capsule to an equilibrium state. The resulting alginate-poor capsule is reacted with a low molecular weight polyamino acid (MW 10-30x $10^3$ ) such a poly-L-lysine (PLL) or chitosan (deacetylated chitin; MW 240x $10^3$) to produce an interacted, less permeable membrane (MW cut-off 40-80x$10^3$). The dual membrane encapsulated cells are then cultured in E-MEM for two to three weeks as described above.

While reference has been made specifically to sodium alginate beads, it will be appreciated by those skilled in the art that any non-toxic water soluble substance that can be gelled to form a shape-retaining mass by a change in conditions in the medium in which it is placed may be employed. Such gelling material generally comprises several chemical moieties which are readily ionized to form anionic or cationic groups so that the surface layers can cross link to form a permanent membrane when exposed to oppositely charged polymers. Most polysaccharide gums, both natural and synthetic, can be cross-linked by polymers containing positively charged reactive groups such as amino groups. The cross-linking biocompatible polymers which may be reacted with the sodium alginate gum include polylysine and other polyamino acids. The degree of permeability of the membrane formed may be controlled by careful selection of a polyamino acid having the desired molecular weight. Poly-L-lysine (PLL) is the preferred polymeric material but others include chitosan and polyacrylate. Molecular weights typically vary from about $10^4$ to about $10^6$.

The present invention is further illustrated by the following examples. These examples are merely to illustrate aspects of the present invention and are not intended as limitations of this invention.

## EXAMPLE 1

Generation of the heterogeneous fetal CNS-derived parental cells:

SVG cells may be obtained from the American Type Culture Collection, Manassas, VA. (Accession number ATCC CRL 8621). SVG cells were grown by means known in the art. See, U.S. Patent No. 4,707,448 and . Major *et al.*, Proc Natl Acad Sci U S A, 82: 1257-61 (1985).

While immortalized SVG cells were used as a starting material to exemplify the present invention, it is appreciated that immortalized cells, which are cells that have an ability to divide without limit, may be made by a number of means, including immortalization by genetic modification of the cells to express oncogenes such as ras, SV40T-antigen, v-myc, c-myc and cells that have spontaneously immortalized. In addition, cells can be immortalized by genetic modification of the cells to express telomerase (Zhu, Wang *et al.* (1999) Proc Natl Acad Sci U S A 96(7): 3723-8). Immortalized cells include stem cells that have the ability to divide without limit due, at least in part, to their high expression of telomerase. Imortalized cells can be derived from a spontaneous process that is thought to be due to mutation(s). The starting materials of the present invention are not limited therefore to the SVG cells described herein by way of exemplification.

## EXAMPLE 2

Generation of post-crisis immortalized NG1 cells:

SV40 induced-immortalization of human cells was a two step process. The first step was an extension of lifespan in culture, where cells underwent a limited number of doublings beyond the point normal cells undergo senescence. After the lifespan extension, the cells entered "crisis", a state in which cell division was balanced by cell death. The second step involved the rare appearance of a colony of immortalized cells, which were subcultured indefinitely . Bryan & Reddel, Crit Rev Oncog, 5: 331-57 (1994). After SVG cells were cultured to approximately passage 33-38, they entered "crisis" typical of cells expressing SV40, where there was very little cell growth and the cells looked very large and granular. When SVG cells were in crisis, there was microscopically observable cell death. When cells in crisis were fed twice weekly and kept

at a cell density $\geq$ 1.5x106/T75 flask, a subpopulation of cells sometimes emerged from crisis and began growing. Cells of this SVG post-crisis subline are growth immortalized cells and are thus considered a distinct cell population (NG1) cells which were thus generated.

SVG cells were grown in standard medium consisting of Eagle Minimum Essential Medium (EMEM; BioWhittaker Cat# 12-125F) + 10% FBS (BioWhittaker Cat# 14-501F) + 2mM l-glutamine (Quality Biological Cat# 118-084-060). Passaging of the cells was done by removal of the media and washing 2x with Hanks' balanced salt solution (HBSS: BioWhittaker Cat#10-543F), 3 minute incubation with 0.25% trypsin (BioWhittaker Cat#17-161E) to dissociate the cells. The suspended cells were added to fresh standard media and centrifuged at 100x g at room temperature for 5 minutes. The cell pellet was then brought up in fresh standard medium and plated in a new 75 cm2 tissue culture flask.

Pre-crisis SVG cells were subcultured every 3-4 days when the flask was confluent, but during crisis they only needed to be subcultured every 8-10 days due to slow growth and cell death. While in crisis, the SVG cells did not need to be subcultured as frequently, but continue to be seeded at 1.5x106 per 75 cm2 flask. The SVG cells remained in crisis for about 2 months before one or more colonies of cells emerged and began growing in the flask. These colonies, which were mixed together with each passage, outgrew the other cells in the flask and constitute the post-crisis cell line. The NG1-type post-crisis cell line was generated by seeding SVG cells into a designated flask and treating it as a separate cell line or population as it went through and emerged from crisis. The NG1-type cell line is not considered clonal, since it represents a pool of all the colonies that emerged from crisis within the flask.

**EXAMPLE 3**

Preparation of SVG cells for flow cytometric analysis

SVG cells were propagated in standard medium through passage 50 and then trypsinized using 0.25% trypsin in EDTA buffer. These NG1-type cells were plated into tissue culture grade plastic flasks at approximately 1X104 cells/cm$^2$. The cells were allowed incubate for 16 hrs or overnight and then refed with either standard medium or in Neurobasal (NB; Gibco, Gaithersburg, MD) + N2 supplements (Gibco) without serum. The formulation for Neurobasal medium (NB) is available from Life Technologies (1-800-847-4226; Cat. No. 21103) and has been previously described by Brewer *et al.* (J Neurosci Res, 35: 567-576 (1993)) to include (all amounts in parentheses as $\mu$M unless indicated otherwise) inorganic salts ($CaCl_2$ (anhydrous,

1800), $Fe(NO_2)_3.9H_2O$ (0.2), KCl (5360), $MgCl_2$ (anhydrous, 812), NaCl (51300), $NaHCO_3$ (26000) and $NaH_2PO_4.H_2O$ (900)); other components (D-glucose (25000), Phenol Red (23), HEPES (10000) and sodium pyruvate (230)), amino acids (L-alanine (20), L-arganine.HCl (400), L-asparagine.H2) (5), L-cysteine (10), L-glutamine (500), glycine (400), L-histidine.HCl.$H_2O$ (200), L-isoleucine (800), L-leucine (800), L-lycine.HCl (5), L-methionine (200), L-phenylalanine (400), L-proline (67), L-serine (400), L-threoine (800), L-tryptophan (80), L-tyrosine (400), L-valine (800)), and vitamins (D-Ca pantothenate (8), Choline chloride (28), folic acid (8), i-inositol (40), niacinamide (30), pyridoxal-HCl (20), riboflavin (1), thiamine-HCl (10) and vitamin B12 (0.2)). A 100x N2 supplement contains: insulin 500 $\mu$g/ml, human transferrin 10,000 $\mu$g/ml, progesterone 0.63 $\mu$g/ml, putrescine 0.16 $\mu$g/ml, and selenite 0.52$\mu$g/ml.

Forty eight-seventy two hours later the cells were harvested by trypsinization, resuspended in PBS with 0.1% BSA (bovine serum albumin) at 106 cells/ml and processed for FACS analysis according by the protocol previously described . Maric *et al.*, Neuromethods, 33: 287-318 (1999). In order to achieve single cell suspensions for application for flow cytometry, trypsin was used instead of papain to obtain better results.

After isolation, the cells were reacted with appropriate reagents and analyzed by means of a fluorescence activated cell sorter (FACS). Up to five different parameters of each single cell (including cell size and complexity, and immunocytochemical, membrane potential and calcium fluorescence signals) were measured simultaneously, at the rate of several thousand cells per second. In some experiments, precise sorting of different cell populations then followed, based on any one or a combination of these different cell parameters.

**Table 2**: Antibodies and fluorescent labels for CNS cell types

| Primary label | Secondary label |
|---|---|
| Tetanus toxin fragment C (TnTx) with anti-TnTx fragment C antibody (IgG1) | Goat anti-mouse IgG1-PE- CY5 |
| Mouse IgM A2B5 antibody | Goat anti-mouse-IgM-PE |
| Cholera toxin-FITC | |
| Rabbit anti-nestin | Goat anti-rabbit Ig-PE |

The primary labels used for FACS analysis (Figures 1 and Figure 2) included a mixture of 1) TnTx fragment C (Sigma, St Louis, MO) combined with a mouse monoclonal class IgG anti-TnTx fragment C antibody (Boehringer Mannheim, Indianapolis, IN), 2) a mouse monoclonal class IgM antibody A2B5 (Boehringer Mannheim, Indianapolis, IN), 3) cholera toxin-conjugated with FITC (Sigma, St Louis, MO) and/or 4) a rabbit polyclonal anti-nestin rabbit antibody (Messam CA, Hou J, Major EO: Exp Neurol 161:585-596, 2000 "Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody"). These primary labels were used to characterize the cell phenotypes of the present invention.

The secondary labels used for flow cytometry included 1) goat anti-mouse IgG1-PE- CY5 (CALTAG Laboratories, Burlingame, CA), 2) Goat anti-mouse-IgM-PE (Jackson Immunoresearch Labs, West Grove, PA) and 3) Goat anti-rabbit Ig-PE (CALTAG Laboratories, Burlingame, CA). Immunofluorescence characteristics were acquired from 200,000 cells randomly sampled by FACS using a 488 nm laser excitation and fluorescence filters set at 525± 30, 575±25 and 670±20 nm to detect FITC, PE and PE-CY5, respectively.

All measurements were made with a FACSTAR+ flow cytometer (Becton Dickinson Mountain View, CA). Cells were excited using an argon ion laser (Spectra Physics, Model 2016, Mountain View, CA) operated at 500 mW and tuned to 488 nm. Forward angle light scatter (FALS), a property related to cell size, and different fluorescence emissions of individual elements were randomly recorded at 1,000-2,000 events/sec. This rate of data acquisition allowed profiling the properties of ~ 10,000 cells in 5-10 seconds. FALS data were collected in a linear mode using a combination of 488 ± 10 nm bandpass and neutral density filters, while fluorescence emissions were logarithmically amplified and filtered at appropriate wavelengths. In multiple labeling experiments, fluorescence emissions were corrected for color crossover by using electronic compensation. FALS properties and fluorescence intensities were each resolved into 1024 channels. The data were analyzed using Cell Quest Analysis software operating on a FACStation Macintosh-based computer platform (Becton Dickinson, Mountain View, CA).

Specific and preferred reagents and procedures included the use of Normal Physiological Medium (NPM) was prepared with 0.1% BSA,145mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgCl_2$, 10mM Hepes and 10mM glucose 18.02g/100ml 10 ml at pH 7.3 and osmolarity of 290-300 mOsm and filter sterilized. A solution of tetanus toxin fragment C (Sigma) and anti-TnTx (Boehringer Mannheim) was prepared by mixing them together at 1:1 ratio (e.g., add 1 mg TnTx

for every 1 mg of anti-TnTx) and allowing the reaction to occur at 4°C for 30 minutes. Cells were resuspended at a final density of 10 million cells/ml in a NPM solution with BSA (NPM/BSA). One :g TnTx/anti-TnTx mixture was used for every one million cells and incubated at 4°C for 1 hour. The cells were cetrifuged at 200 x g at 4°C for 10 minutes after which supernatant medium was decanted off and the cells resuspended in 10 ml of NPM/BSA. This wash step was repeated twice more after which the cells were resuspended at a final density of 10 million cells/ml in a NPM/BSA. One :g of PE/CY5-conjugated goat anti-mouse IgG1(Caltag Laboratories) and 1 :g FITC-conjugated ChTx were added for every one million cells and incubated at 4°C for 1 hour. The centrifuge and wash steps in NPM/BSA were then repeated three times followed by resuspension of the cells at a final density of 10 million cells/ml in a NPM/BSA.

Cells were double immunolabeled with anti-A2B5 and TnTx antibodies as described and categorized into four populations ($TnTx^+/A2B5^-$, $TnTx^+/A2B5^+$, $TnTx^-/A2B5^+$ and $TnTx^-/A2B5^-$) based on their fluorescence signatures determined by FACS electronic gates. The four populations were sorted by means of electrically charged saline droplets, which were deflected by charged plates directly into appropriate test tubes. Sorted cells were then washed twice in physiological saline and re-analyzed to test for sorting purity, which was greater than 96% in all cases. After sorting, the viability of the cells remained unchanged, with less than 5% Trypan Blue or PI positive (dead or dying) cells in every sorted subpopulation.

## EXAMPLE 4

Effects of eliminating serum in culture conditions

Altering the medium composition for a period of 24-72 hours resulted in reproducible alterations of the relative cell phenotypes. Maintaining the heterogeneous NG1 cell population in standard medium resulted in a relatively high percentage of A2B5+, ChTx+ and TnTx- cells. The A2B5+ cells were decreased from 26% to 5% when the medium for the NG1 cells was changed to Neurobasal + N2 supplements. This was consistent with no significant loss of cells due to the changes in medium. There was a shift to a more neuronal phenotype identified by an increase in the % of ChTx +, TnTx + cells when the cells were in Neurobasal + N2 supplements. The ChTx +, TnTx + cells went from 2% to 18% of the NG1 cells as the medium changed from standard medium to Neurobasal + N2 supplements (Table 3). Consistent with a medium-induced

metabolic change from a glial to a neuronal phenotype, there was a complete loss of JC virus sensitivity when the medium was changed to Neurobasal + N2 supplements.

**Table 3**: NG1 cells express immature neuronal and glial markers dependent on cell culture conditions

| Phenotype | EMEM + 10% FBS (NG1 population) | Neurobasal + N2 supplements (NG3 population) |
|---|---|---|
| ChTx+ | 97% | 99% |
| ChTx+, TnTx+ | 2% | 18% |
| A2B5+ | 23% | 7% |
| ChTx -/TnTx - | | 97% are nestin+ |
| JC virus sensitive | 35-40% | 0% |

ChTx: Cholera toxin

TnTx: Tetanus toxin

A2B5: glial or neuronal progenitor

JC virus infects cells of glial lineage

**EXAMPLE 5**

Demonstration that there are stem/progenitor cells identified from the heterogeneous population:

The most undifferentiated multipotent cells would be expected to be the cells that do not express any of the markers specific for glial or neuronal lineage. These negative cells (ChTx -, TnTx -, A2B5-) cells were tested for their possible "stem/progenitor" status. Multi-potential stem/progenitor cells can be identified by expression of the intermediate filament protein nestin . Lendahl *et al.*, Cell, 60: 585-95 (1990). About 95% of the TnTx-/ChTx- cells labeled positive for nestin (Table 2 and Figure 3). Consistent with the existence of a multipotent cell population, there was a dramatic loss of JC virus sensitivity in the Neurobasal + supplements medium compared to the standard medium (Table 2). This confirms that there is a stem/progenitor cell population in the NG1 cell line that is present.

While the use of TnTx, ChTx and A2B5 as markers is preferred according to the present invention, the populations of cells according to the invention are not limited to being characterized by only these markers. Specifically, other cellular phenotypic markers or binding

partners, such as the presence or absence of cell surface receptors, neurotransmitter transporters, ion channels, and/or ion exchange pumps, for example, may further characterize the developmental stage of the cells and/or cell populations of the present invention. Such further markers are known to those of ordinary skill and have been described in, for example, Siegel 1994; Palmer, Takahashi *et al.* 1997; Sah, Ray *et al.* 1997; Snyder, Yoon *et al.* 1997; Gage 1998; Gage, Kempermann *et al.* 1998; Ling, Potter *et al.* 1998; Wagner, Akerud *et al.* 1999; and Gage 2000. Specifically, for example, neurons can be identified by the expression of N-type calcium channels. Oligodendrocytes can be identified by the presence of Galactocerebroside C (GalC) on the cell surface. Even more specific markers for subpopulations of neurons may include the use of antibodies to receptors such as the μ opioid receptor to obtain cells involved in the sensory/pain pathway. FACS can be performed on greater than three labels simultaneously, which allow sorting of cells based on the number of labels that can be resolved in the emission spectrum.

**EXAMPLE 6**

The lack of effect of forskolin on the phenotypic marker expression:

The effect of adenylate cyclase activator forskolin was tested on the NG1 cell phenotype. There was no effect of a 24 hour treatment period with forskolin (5 $\mu$M) on the resulting NG3 cell phenotypic expression of ChTx, TnTx or A2B5 whether they were in EMEM + 10% FBS or in Neurobasal + N2 supplements (Figure 1).

**EXAMPLE 7**

Characterization and isolation of distinct cell types.

Results of A2B5 and/or TnTx and/or ChTx and/or nestin double or triple immunolabeling reactions with the NG1 cells reveals qualitative and quantitative differences in expressions and co-expressions of these surface epitopes are presented in Figures 1-2. The following distinct cell types observed from the flow cytometry are listed below:

TnTx-/ChTx- (1)

TnTx-/ChTx+ (2)

TnTx+/ChTx- (3)

TnTx+/ChTx+ (4)

A2B5-/TnTx- (5)

A2B5+/TnTx+ (6)

A2B5+/TnTx- (7)

A2B5-/TnTx+ (8)

A2B5-/ChTx- (9)

A2B5+/ChTx+ (10)

A2B5-/ChTx+ (11)

A2B5+/ChTx- (12)

Type 1 cells above can be further divided into nestin+ (Type 13) and nestin- (Type 14) cells that are also TnTx-/ChTx-.

The cells identified above represent distinct cell populations that can be sorted and possibly cultured for further use. There may be uses for an optimal mixture of 2 or more populations of these cells. Standard methods of culturing cells of the CNS are established. Banker & Goslin, Cellular and Molecular Neuroscience, (1991); Brewer *et al.*, J Neurosci Res, 35: 567-576 (1993); Freshney Culture of Anima Cells, Wiley Liss, NY, NY 3rd Ed. 1994, and are also indicated in other references cited herein. There are numerous medium formulations that can be obtained commercially (Gibco, Gaithersburg, MD) and used for culturing cells of the CNS. Medium formulations could also be supplemented to include growth factors and cytokines including basic or acidic fibroblast growth factor, glial cell line derived neurotrophic factor, leukemia inhibitory factor, interleukins that are commercially available (Gibco, Gaithersburg, MD; R&D Systems, Minneapolis, MN; Sigma, St. Louis, MO) preferably including IL1 , IL1∀ , IL1∃, insulin, insulin-like growth factors, ciliary neurotrophic factor, brain-derived neurotrophic factor, neurotrophin 4, neurotrophin 3, bone morphogenic proteins, nerve growth factor, epidermal growth factor, platelet derived growth factor, sonic hedgehog proteins and any other commercially available proteins that are known to support survival, proliferation of cells in the CNS.

**EXAMPLE 8**

Flow cytometric sorting of distinct populations of cells

The studies of specific cell populations of the CNS *in vitro* are complicated by limited abilities to unequivocally identify and expeditiously isolate pure cell types. Investigators have commonly used selective culture conditions to isolate neurons, astrocytes, oligodendrocytes and other cell types. However, these methods usually require several days to weeks of culturing,

during which time cell properties may change and no longer reflect those expressed *in vivo*. A variety of positive and negative selection methods now exist that permit enrichment of specific populations based on surface epitopes (i.e., panning and complement lysis). However, these are complicated by the fact that many antigenic epitopes are shared among different cell types during development and hence a combination of markers is required for the identification and isolation of specific cell populations. Using flow cytometers equipped for sorting, it is possible to isolate very pure specific cell populations based on the presence or absence of multiple phenotypic or functional cell markers and even the relative intensity of these markers.

Sorting of cells can be based on the expression or lack of surface epitopes. Cells were double or triple immunolabeled with anti-A2B5 and/or cholera toxin and/or TnTx and/or nestin antibodies, as described previously and categorized into 14 populations based on their fluorescence signatures determined by FACS electronic gates. Based on the information cited, the 14 cell types indicated above, and mixtures thereof, could be sorted by means of electrically charged saline droplets, which could be deflected by charged plates directly into appropriate test tubes . Maric *et al.*, Neuromethods, 33: 287-318 (1999).

**EXAMPLE 9**

JC Virus infection of SVG cells cultured in standard medium or in Neurobasal medium + N2 supplements

JC Virus has a tropism for cells of a glial phenotype in the human central nervous system. JCV does not multiply in cells of a neuronal phenotype either in culture or *in situ* in the human brain . Atwood *et al.*, J Neurovirol, 1: 40-9 (1995; Major *et al.*, Clin Microbiol Rev, 5: 49-73 (1992).

NG1 cells at passage 50 were trypsinized from semiconfluent monlayers and plated into multiple 75cm flasks at 1X106 cells/flasks or on glass coverslips in 35mm wells. The cells were allowed to attach overnight in E-MEM medium with 10% FBS. Half of the flasks or coverslips were then refed with Neurobasal medium with N2 supplements and the other half were refed with fresh standard medium. All cultures received 25ug/ml gentamicin as an antibiotic.

48 hrs later, the cells were treated with JC Virus mad –4 strain preparation at a multiplicity of infection of 0.1-1.0 infectious particle per cell by adsorption for 90 minutes, and then refed with either EMEM or with Neurobasal medium with N2 supplements.

The cultures were incubated at 37°C for 7 days after which the cells and the supernatant fluids were harvested and processed for hemaglutination assays (HA) to measure virus multiplication. HA assays were conducted using human type O erthryocytes in 2 fold serial dilutions according to the previously published protocol. [Neel et al., Proc Natl Acad Sci 93:2690-2695 (1996).

The coverslips were washed in PBS, fixed with acetone/methanol and used for immunocytochemistry for JC viral antigens. Fixed cells were treated with rabbit polyclonal antiserum to the JCV capsid antigen for 2 hrs at 37°C, washed and treated with mouse fluorescein conjugatged anti-rabbit IgG. The cells were examined using a Zeiss inverted ICM 405 microscope with Xenon arc lamp and appropriate filters for UV light examination.

Only the flasks that were cultured on E-MEM with 10% FBS showed a positive HA titer of 1:256 per ml at 7 days of infection and also showed 35-40% of the cells infected as determined by capsid antigen expression using the anti JCV capsid antibody. This percentage of JCV capsid positive cells agrees with earlier data on similarly treated NG1 cells . Hou & Major, J Neurovirol. 4: 451-456 (1998). The cells cultured on Neurobasal medium with N2 supplements did not have either a HA titer or show any immunofluorescence using the anti JCV antibody. Therefore, the SVG cells were metabolically shifted to a more neuronal phenotype (increased TnTx+ and decreased A2B5+), they were no longer capable of supporting JC Virus multiplication.

## EXAMPLE 10

NG1 and NG3 cells secrete different levels of trophic factors

SVG-derived cells are able to secrete numerous trophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), transforming growth factor β1 (TGFβ1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF-2; also known aa basic FGF). Table 4 indicates that the SVG cell secretion of trophic factors changes when the medium is Neurobasal + N2 compared to EMEM + 10% FBS. Specifically, there is a decreased secretion of BDNF in Neurobasal + N2 medium compared to EMEM + 10% FBS. The secretion of BDNF in EMEM without FBS was nearly identical (34.1) to the secretion in EMEM/FBS. Therefore, the higher level of BDNF in EMEM + 10% FBS is not due to BDNF in the serum. This trophic factor

secretion data provides a further phenotypic distinction for SVG-derived cells based on the Neurobasal + N2 medium compared to EMEM + 10% FBS cell culture medium.

**Table 4.** NG1 and NG3 cells secrete different levels of trophic factors

| Trophic Factor | Medium | Initial Cell Count | Final Cell Count | pg Trophic Factor/$10^5$ cells |
|---|---|---|---|---|
| BDNF | EMEM + 10%FBS | 500,000 | 1,005,000 | 32.6 |
| | Neurobasal + N2 | 500,000 | 720,000 | 5.0 |
| | EMEM + 10%FBS | 200,000 | 232,000 | 46.5 |
| | Neurobasal + N2 | 200,000 | 217,000 | 2.4 |
| | EMEM + 10%FBS | 100,000 | 135,000 | 36.0 |
| | Neurobasal + N2 | 100,000 | 52,000 | 0.0 |
| NGF | EMEM + 10%FBS | 500,000 | 1,005,000 | 33.4 |
| | Neurobasal + N2 | 500,000 | 720,000 | 24.5 |
| | EMEM + 10%FBS | 200,000 | 232,000 | 54.0 |
| | Neurobasal + N2 | 200,000 | 217,000 | 33.8 |
| | EMEM + 10%FBS | 100,000 | 135,000 | 58.2 |
| | Neurobasal + N2 | 100,000 | 52,000 | 31.0 |
| VEGF | EMEM + 10%FBS | 500,000 | 1,087,000 | 95.7 |
| | Neurobasal + N2 | 500,000 | 825,000 | 67.6 |
| | EMEM + 10%FBS | 200,000 | 365,000 | 194.9 |
| | Neurobasal + N2 | 200,000 | 295,000 | 84.5 |
| | EMEM + 10%FBS | 100,000 | 185,000 | 30.2 |
| | Neurobasal + N2 | 100,000 | 188,000 | 22.9 |
| PDGF | EMEM + 10%FBS | 500,000 | 1,087,000 | 44.4 |
| | Neurobasal + N2 | 500,000 | 825,000 | 23.1 |
| | EMEM + 10%FBS | 200,000 | 365,000 | 33.3 |
| | Neurobasal + N2 | 200,000 | 295,000 | 30.9 |
| | EMEM + 10%FBS | 100,000 | 185,000 | 58.4 |
| | Neurobasal + N2 | 100,000 | 188,000 | 27.5 |

ELISA results from media conditioned with NG1 cells (EMEM + 10% FBS) or NG3 cells (Neurobasal + N2). Cells were grown in 6-well plates (35-mm well diameter) in 0.9 ml of media per well. Cytokine concentrations are pg/$10^5$ cells x 48 hrs. "0" represents optical densities below the detectable threshold of the assay. The assays were performed according to the instructions of the supplier using VEGF and PDGF ELISA kits from R&D Systems (Minneapolis, MN) and BDNF and NGF ELISA kits from Promega (Madison, WI). These suppliers also have ELISA kits for NT-3, TGFβ1 and FGF-2.

**EXAMPLE 11**

NG1 and NG3 cells can be distinguished morphologically

A characteristic of NG1 cells (EMEM + 10% FBS) is their flat epitheloid morhology (Figure 4A). However, NG3 cells (Neurobasal + N2 = NB + N2) form neurosphere-like clusters (Figure 3B). Normal human neural progenitor (NHNP) cells (Clonetics, Frederick, MD) are multipotent cells that can differentiate into neuronal or glial cells. These NHNP cells (Figure 3C) appear morphologically identical to the NG3 cells as clusters of cells. The morphological characteristics of the NG3 cells are thus consistent with human multipotent stem/progenitor cells as observed previous and termed neurospheres (Reynolds and Weiss, Science, 255:1707-1710 (1992)). It was surprising that the NG3 cells do not require mitotic factors EGF or FGF-2 to form these neurosphere-like clusters and that the NG3 cells maintain a proliferative state. These neurosphere-like clusters were formed after culture in NB + N2 for a period greater than 30 days.

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All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

**We claim:**

1. An isolated central nervous system cell line comprising an immortal multipotent cell having the potential to differentiate toward a neuronal cell or a glial cell.

2. The cell line according to claim 1 wherein said central nervous system derived cell line is derived from a human central nervous system.

3. An isolated multipotent cell of claim 1.

4. A cell of claim 3 which is characterized by a marker combination selected from the group consisting of TnTx-/ChTx-, TnTx+/ChTx+, TnTx+/ChTx-, TnTx+/ChTx+, A2B5-/TnTx-, A2B5+/TnTx-, A2B5-/TnTx+, A2B5-/ChTx-, A2B5+/ChTx+, A2B5-/ChTx+, A2B5+/ChTx-, TnTx-/ChTx-/nestin+, and TnTx-/ChTx-/nestin-

5. An isolated cell or tissue derived from the cell line of claim 1.

6. A cell according to claim 3 further comprising a heterologous nucleic acid sequence which encodes a biologically active peptide or protein.

7. A cell according to claim 6, wherein said biologically active peptide or protein is a disease associated peptide or protein.

8. A cell according to claim 6 wherein said biologically active peptide or protein is an enzyme, a trophic factor, a cytokine or a disease associated antigen.

9. A cell according to claim 8 wherein said enzyme is selected from the group consisting of tyrosine hydroxylase, GTPCH1, AADC and VMAT2; said trophic factor is selected from the group consisting of GDNF, VEGF, BDNF, NGF, bFGF, TGFβ, CNTF, PDGF, BMP, LIF, Neurturin, Persephin, Neublastin, NT4/5, NT3, Midkine; said cytokine is selected from the group consisting of IL-10 and IL-6.

10. A cell according to claim 8 wherein said nucleic acid is operably linked to a transcriptional promoter.

11. A cell according to claim 8 wherein said nucleic acid is operably linked to a regulatable promoter system.

12. An isolated or purified cell population comprising a cell of claim 3.

13. The cell population of claim 12, wherein said population is selected from the group consisting of an NG1, NG2, and NG3 populations of cells.

14. A method of identifying a multipotent cell comprising measuring for the presence or absence of a cell-derived binding partner for TnTx, a cell-derived binding partner for ChTx and, optionally, a cell-derived binding partner for an A2B5 antibody in a cell sample which is believed to contain a multipotent cell.

15. The method according to claim 14, wherein said multipotent cell is a fetal central nervous system derived cell.

16. The method of claim 13 wherein said method comprises mixing said sample with at least one factor which specifically binds to at least one of a cell-derived binding partner for TnTx, a cell-derived binding partner for ChTx and a cell-derived binding partner for an A2B5 antibody, under conditions where said at least one factor binds to said cell, and detecting said binding, wherein said binding indicates the presence of said multipotent cell.

17. The method of claim 16 wherein said factor which binds the A2B5 binding partner is an A2B5 antibody or a fragment thereof; said factor which binds a binding partner of ChTx is a ChTx binding partner antibody or a fragment thereof or ChTx or a fragment thereof; and said factor which binds a binding partner of TnTx is a TnTx binding partner antibody or a fragment thereof or TnTx or a fragment thereof.

18. The method of claim 14 further comprising mixing said sample with a factor which specifically bind to human nestin and detecting whether said ligand binds to nestin in said sample.

19. The method of claim 14, wherein said ligand or ligands contain a detectable label and, optionally, said mixing further comprises addition of a further detectable component which binds to said ligand.

20. The method of claim 19 wherein, said detectable label is fluorescent.

21. The method of claim 20, wherein said detection further comprises analyzing said cells with a fluorescence activated cell sorter.

22. A method of purifying a multipotent cell comprising separating a cell identified according to claim 14.

23. A method of enriching a population of cells with said multipotent fetal nervous system derived cells comprising culturing said population in the presence of serum followed by culturing said population in a non-serum containing media.

24. The method of claim 23, wherein said population is passaged in serum containing media through crisis.

25. A method of enriching a population of cells containing multipotent fetal nervous system derived cells with said multipotent cells comprising passaging said population in serum containing media through crisis wherein said population which emerges from crisis is enriched with said multipotent cells.

26. A population of cells produced by the method of claim 23.

27. A population of cells produced by the method of claim 24.

28. A population of cells produced by the method of claim 25.

29. The method of claim 23, wherein said population is derived from the SVG cell line.

30. The method of claim 24, wherein said population is derived from the SVG cell line.

31. The method of claim 25, wherein said population is derived from the SVG cell line.

32. A method of treating a mammal having a neurological syndrome or disease comprising implanting into said mammal a therapeutically effective amount of a composition comprising at least one cell according to claim 4.

33. An isolated cluster of cells comprising a cell of claim 3.

34. A cluster of claim 33 in the form of a neurosphere.

# Immunoidentification of SVG P50 Cells Cultured in FCS-Containing or Serum-Free Media: Comparison Between Control and Forskolin-Treatment (Gated on Live Cells)

## 10 % FCS Culture Medium

Control

Forskolin-Treated

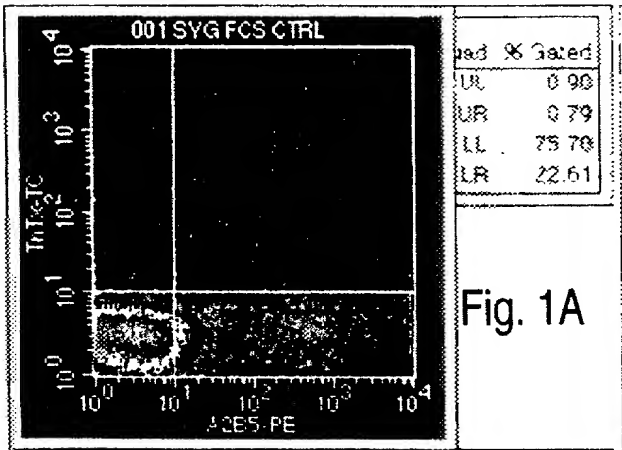



Fig. 1A

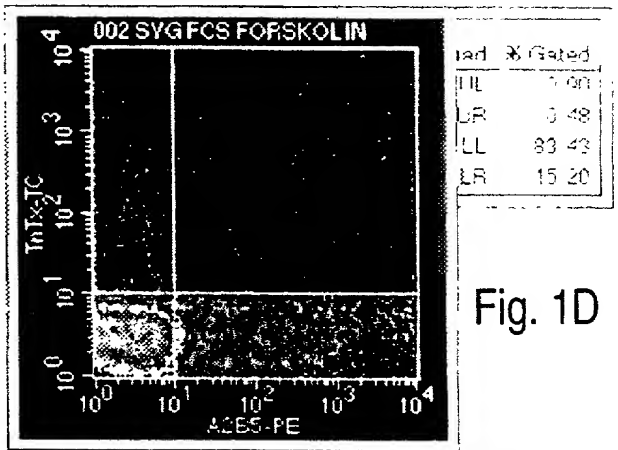



Fig. 1D

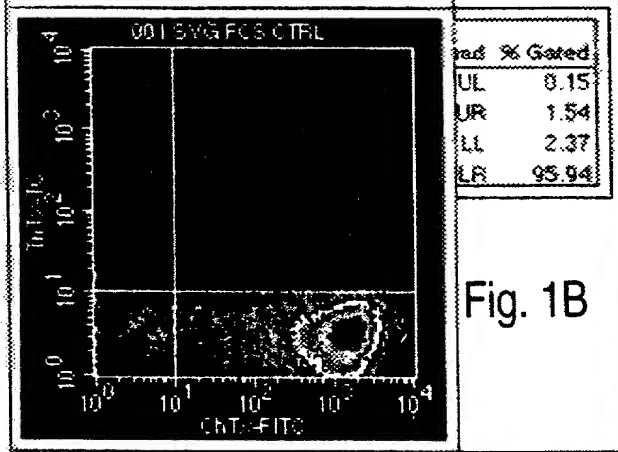



Fig. 1B

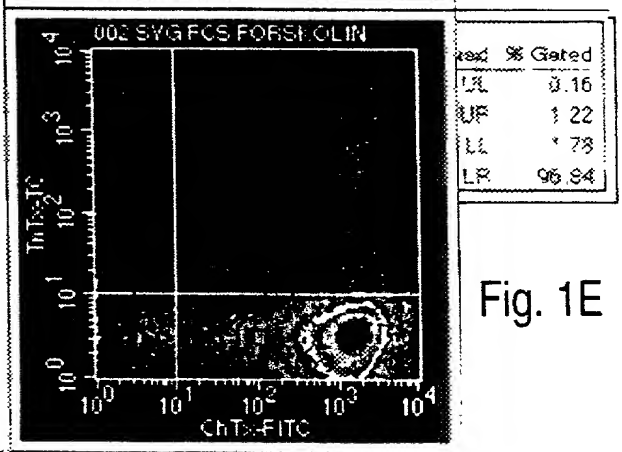



Fig. 1E

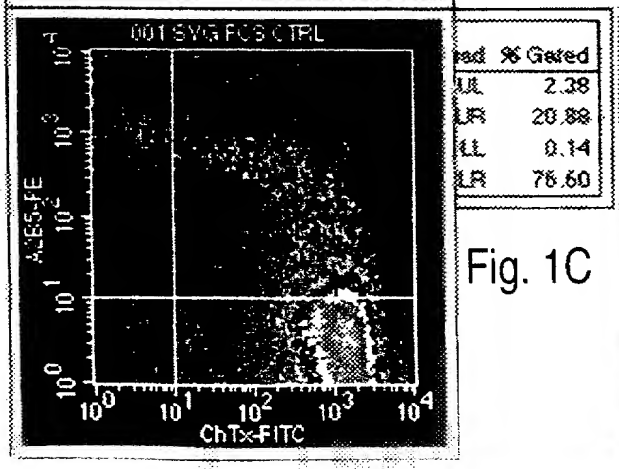



Fig. 1C

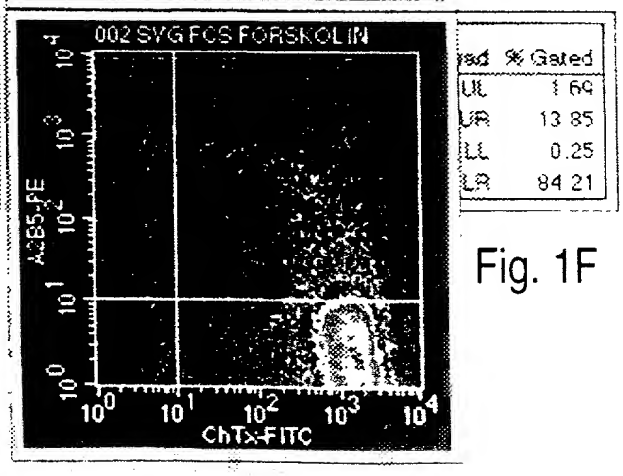



Fig. 1F

# Immunoidentification of SVG P50 Cells Cultured in FCS-Containing or Serum-Free Media: Comparison Between Control and Forskolin-Treatment (Gated on Live Cells)

## Neurobasal/N2 Culture Medium

Control

Forskolin-Treated

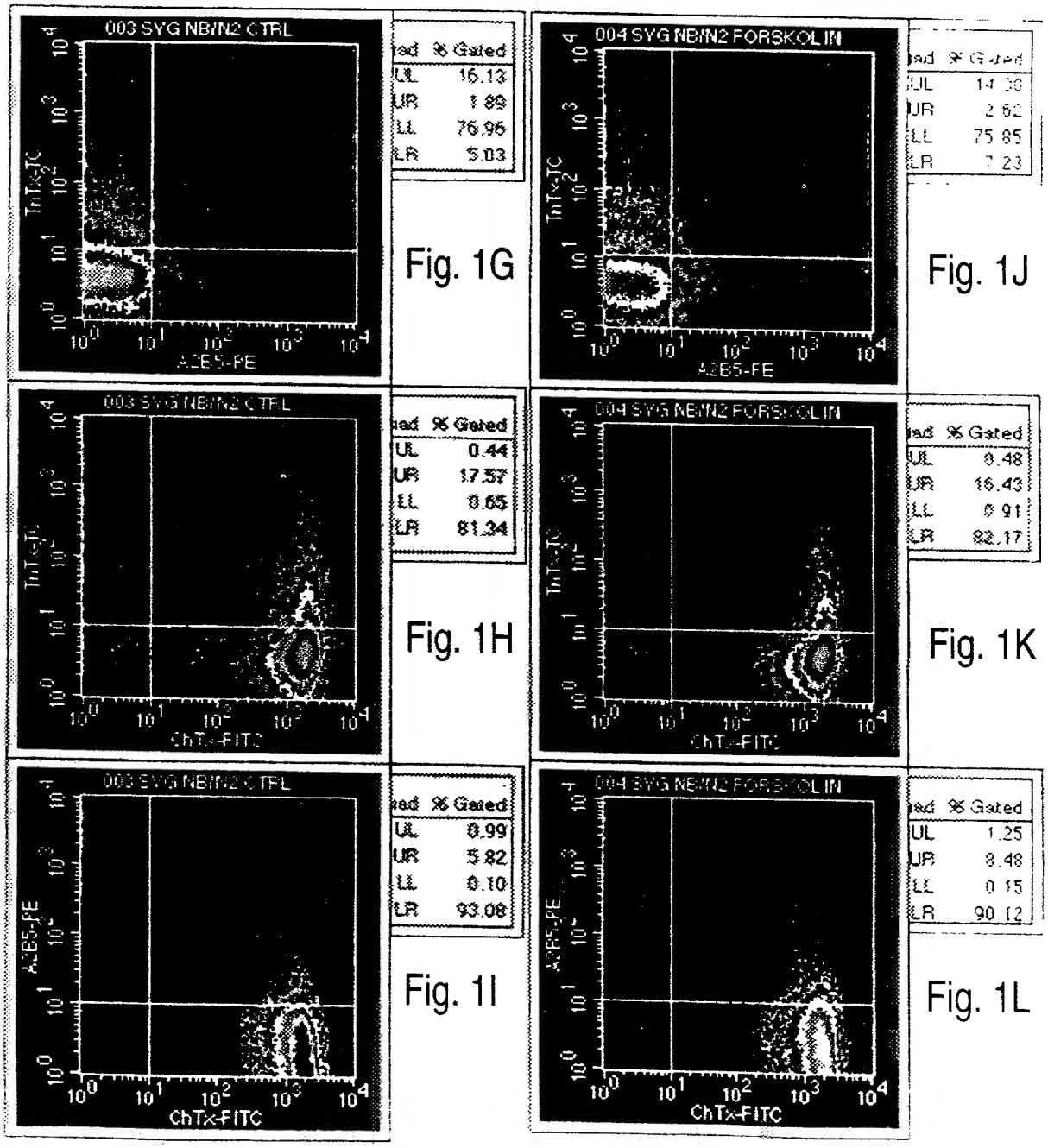



Fig. 1G

Fig. 1J

Fig. 1H

Fig. 1K

Fig. 1I

Fig. 1L

# Immunoidentification of SVG P50 Cells Cultured in Serum-Free Media: (Anti-ChTx-FITCx Anti-TnTx-PE/CY5 x Anti-Nestin-PE)

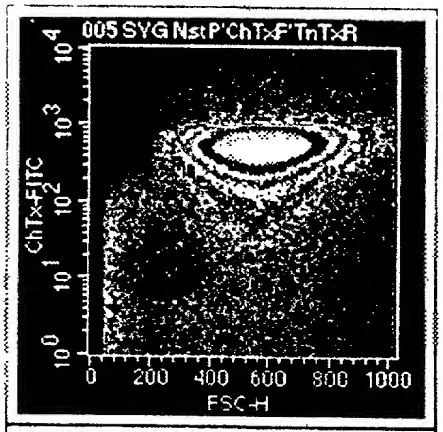



Fig. 2A

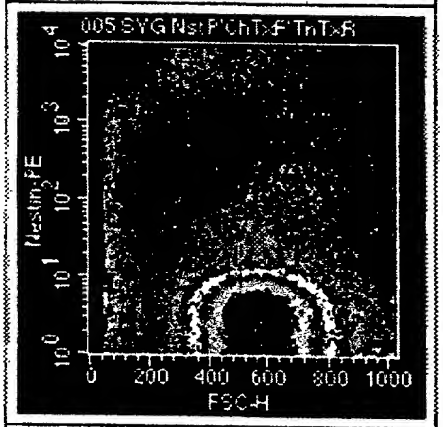



Fig. 2B

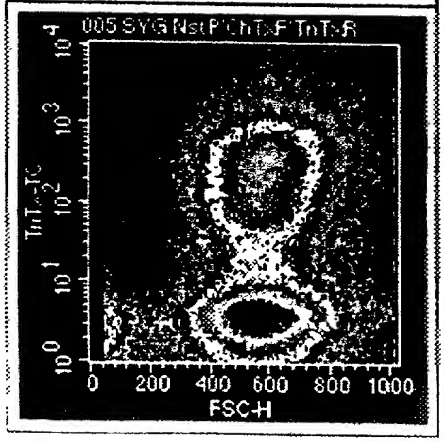



Fig. 2C

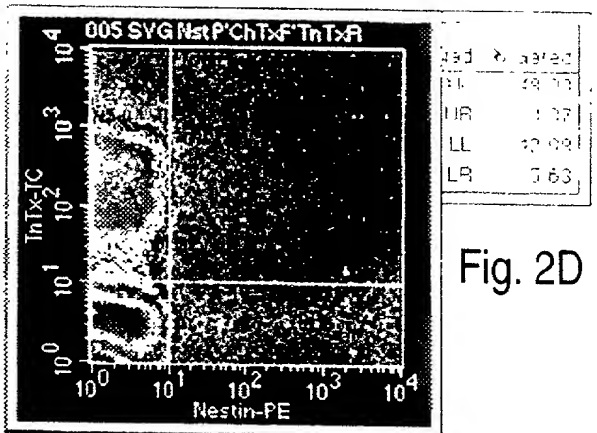



Fig. 2D

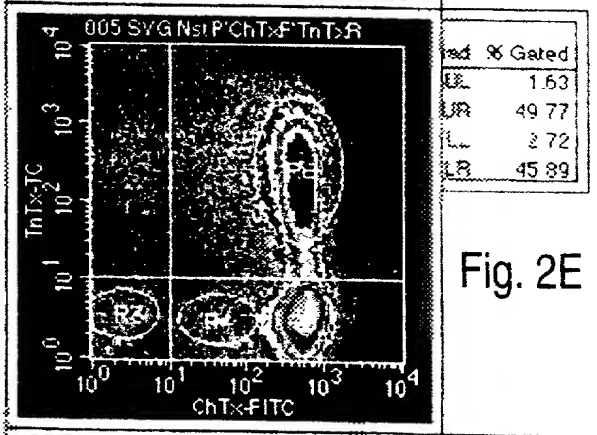



Fig. 2E

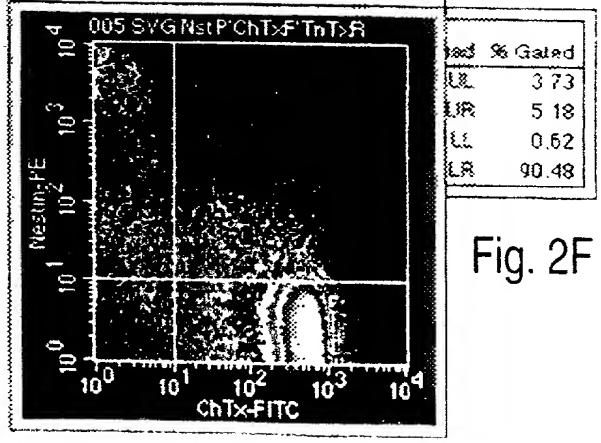



Fig. 2F

Immunoidentification of SVG P50 Cells Cultured in Serum-Free Media: (Anti-ChTx-FITCx Anti-TnTx-PE/CY5 x Anti-Nestin-PE)

Fig. 2G

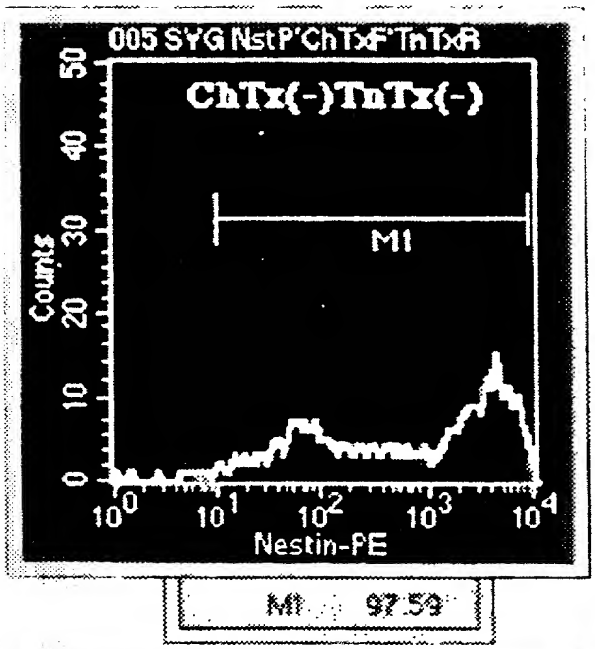



Fig. 2I

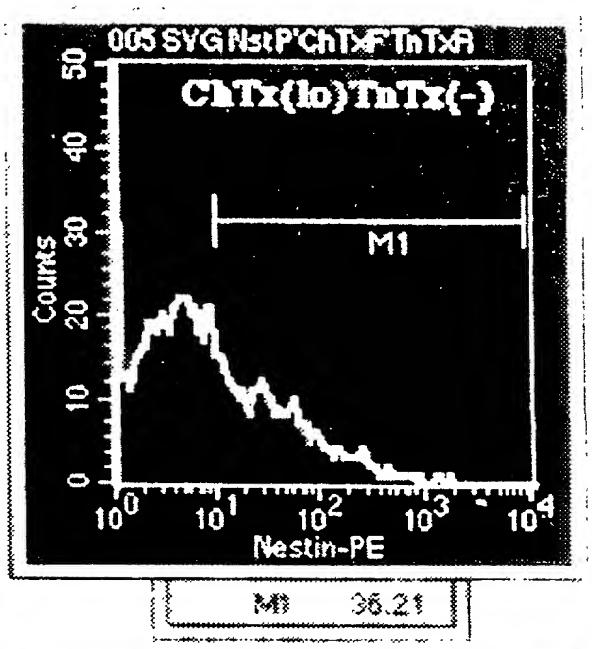



Fig. 2H

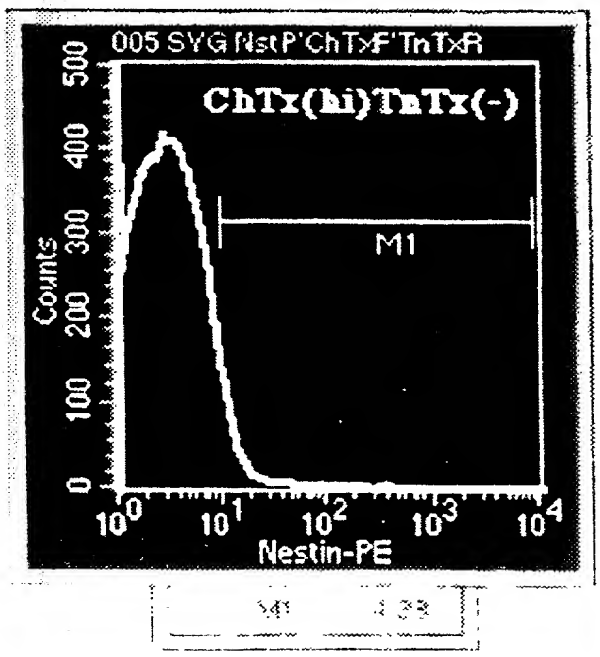



Fig. 2J

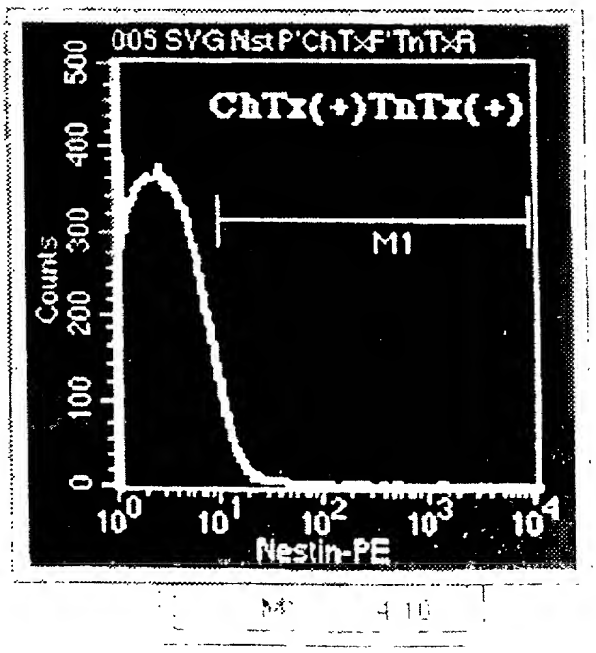


Fig. 3A


Fig. 3B

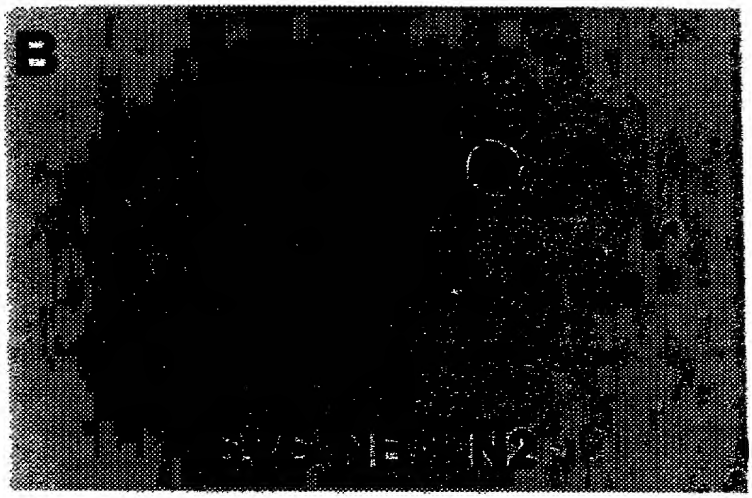
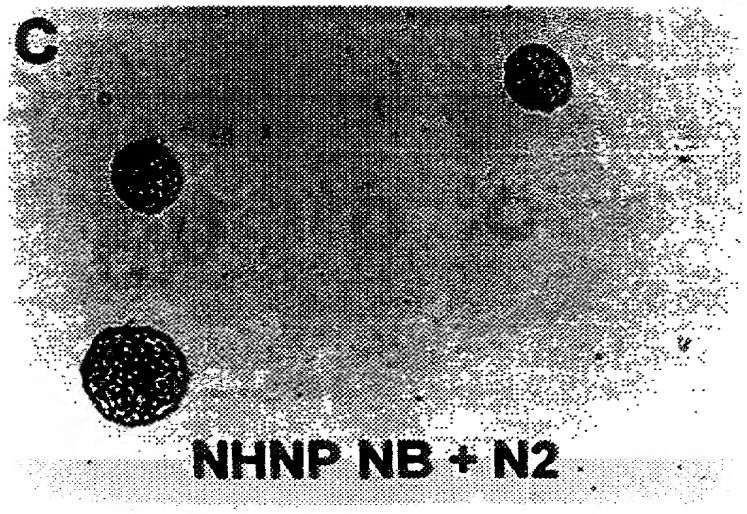



Fig. 3C



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Nixon & Vanderhye P.C. (10/99)
(Domestic Non-Assigned/Foreign) Page 1

#4

# RULE 63 (37 C.F.R. 1.63)
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the specification of which (check applicable box(s)):

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| PCT/US00/06940 | 17 March 2000 | |

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur R. Crawford, 25327; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comuntzis, 31097; Gary T. Tanigawa, 43180. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

1. Inventor's Signature: Eugene O. Major Date: 1-18-02
Inventor: Eugene (first) O. MI Major (last) U.S. (citizenship)
Residence: (city) McLean (state/country) Virginia VA
Mailing Address: 1452 Hampton Hill Circle, McLean, Virginia
(Zip Code) 22101

2. Inventor's Signature: Date:
Inventor: Jeffery (first) L. MI Barker (last) U.S. (citizenship)
Residence: (city) Bethesda (state/country) Maryland
Mailing Address: 5811 Bradley Boulevard, Bethesda, Maryland
(Zip Code) 20814

☒ *See attached sheet(s) for additional inventor(s) information!!*

3. Inventor's Signature: _____ Date: _____
Inventor: Dragan (first) MI Maric (last) U.S. (citizenship)
Residence: (city) Gaithersburg (state/country) Maryland
Mailing Address: 750 Quince Orchard Boulevard, Gaithersburg, Maryland
(Zip Code) 20878

4. Inventor's Signature: _____ Date: _____
Inventor: Harvey (first) MI Rabin (last) U.S. (citizenship)
Residence: (city) Rockville (state/country) Maryland
Mailing Address: 11021 Ralston Road, Rockville, Maryland
(Zip Code) 20852

5. Inventor's Signature: _____ Date: _____
Inventor: Abby (first) MI Sandler (last) U.S. (citizenship)
Residence: (city) Silver Spring (state/country) Maryland
Mailing Address: 2901 Mozart Drive, Silver Spring, Maryland
(Zip Code) 20904

FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.

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Nixon & Vanderhye P.C (10/99)
(Domestic Non-Assigned/Foreign) Page 1

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1. Inventor's Signature: _____ Date: _____

| Inventor: | Eugene (first) | O. MI | Major (last) | U.S. (citizenship) |
|---|---|---|---|---|
| Residence: (city) | McLean | | (state/country) Virginia | |
| Mailing Address: | 1452 Hampton Hill Circle, McLean, Virginia | | | |
| (Zip Code) | 22101 | | | |

2-002. Inventor's Signature: Jeffery L. Barker Date: 1/14/2002

| Inventor: | Jeffery (first) | L. MI | Barker (last) | U.S. (citizenship) |
|---|---|---|---|---|
| Residence: (city) | Bethesda | | (state/country) Maryland MD | |
| Mailing Address: | 5811 Bradley Boulevard, Bethesda, Maryland | | | |
| (Zip Code) | 20814 | | | |

☒ *See attached sheet(s) for additional inventor(s) information!!*

3. Inventor's Signature: Dragan Maric Maric Date: 1/14/2002
Inventor: Dragan (first) MI Maric (last) U.S. (citizenship)
Residence: (city) Gaithersburg (state/country) Maryland MD
Mailing Address: 750 Quince Orchard Boulevard, Gaithersburg, Maryland
(Zip Code) 20878

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Residence: (city) Rockville (state/country) Maryland
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Residence: (city) Silver Spring (state/country) Maryland
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(Zip Code) 20904

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#24

2370-67

Nixon & Vanderhye P.C. (10/99)
(Domestic Non-Assigned/Foreign) Page 1

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558951

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Residence: (city) Gaithersburg (state/country) Maryland

Mailing Address: 750 Quince Orchard Boulevard, Gaithersburg, Maryland

(Zip Code) 20878

4. Inventor's Signature: Harvey Rabin Date: 11/20/01

Inventor: Harvey (first) MI Rabin (last) U.S. (citizenship)

Residence: (city) Rockville (state/country) Maryland MD

Mailing Address: 11021 Ralston Road, Rockville, Maryland

(Zip Code) 20852

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|---|---|---|
| PCT/US00/06940 | 17 March 2000 | |

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur R. Crawford, 25327; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comuntzis, 31097; Gary T. Tanigawa, 43180. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

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☒ *See attached sheet(s) for additional inventor(s) information!!*

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**FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.**

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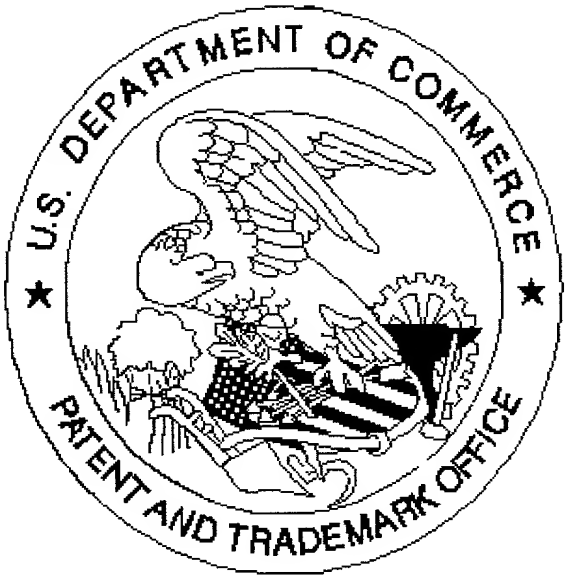




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